Claim 1. Claims 3 and 4 have been rejected under 35 U.S.C. § 102(b) as allegedly anticipated by Masuno et al. or Bast et al. Claims 5-10 have been rejected under 35 U.S.C. § 103 as describing subject matter allegedly rendered obvious by the teachings of Masuno et al. and Bast et al. in view of Pestka or the WO patent 85/00663.

In response to the rejection, applicant has amended the claims which together with the comments herein are deemed to place this case in condition for allowance. Favorable reconsideration of all the pending claims is respectfully requested.

In response to the objection, Applicant has amended Claim 1 by deleting the phrase "substantially purified" therefrom. This amendment is deemed to overcome the Examiner's objection and place Claim 1 in condition for allowance.

With respect to the Examiner's rejection under 35 U.S.C. §102(b), Masuno et al. and Bast et al. disclose monoclonal antibody OC 125 which recognizes a determinant on the antigen CA 125. The Examiner alleges that OC 125 is identical to the antibodies of the instant invention because of the inherent characteristics of the CA 125 antigen. The characteristics of CA 125 as disclosed in the prior art are summarized below.

Masuno et al. analyzed the antigen recognized by OC 125 (i.e., the CA 125 antigen) in culture supernatents of OVCA 432 and OVCA-433 cells (epithelial ovarian carcinoma lines) and showed that CA 125 eluted in the void volume when chromatographed on Sephadex G-200 (see Chart 4 on Page 2816, Column 2 and the section entitled "Detection of Shed Antigen in Spent Culture Medium" at Column 1 of the same page). Sephadex G-200 excludes globular proteins having molecular weights

>600,000 (see attachment). Since CA 125 is excluded from the Sephadex G-200 gel, it is likely to be a high molecular weight complex or molecule having an $M_r$ >600,000. A skilled artisan recognizes this exclusion behavior does not establish whether CA 125 is a single protein species or a stable complex of several smaller proteins (or subunits).

In addition, Masuno et al. found that the CA 125 antigen immunoprecipitated by OC 125 from cells harvested by trypsinization (i.e., enzymatic means) had protein bands at an $M_r$ of 180,000 and less than <20,000 on SDS-PAGE. In contrast, immunoprecipitates from cells harvested by mechanical means (i.e., scraping) did not enter the stacking gel during SDS-PAGE, indicating a high molecular weight complex or molecule and consistent with CA 125 having a molecular weight >600,000 (see section entitled "Analysis of Cell Surface Antigens on SDS-PAGE," Page 2816, Column 2).

Bast et al. state that CA 125 is a high molecular weight glycoprotein (Page 883, the sentence bridging Columns 1 and 2). They do not disclose a specific molecular weight for this glycoprotein.

For purposes of discussion, CA 125 will be considered as a multimeric complex rather than a single species. This usage is not intended to exclude the possibility that CA 125 consists of a single protein species. The use of "subunit" refers to a protein that forms part of the multimeric complex or, if CA 125 is a single species (or single protein), then subunit refers to a subspecies or domain of the protein.

Thus, Masuno et al. and Bast et al. teach that the CA 125 antigen is a high molecular weight complex ($M_r$ > 600,000) having at least one subunit of an about 180,000 kDa. Further, the art teaches that the antigenic determinants (or epitopes)

recognized by the monoclonal antibody OC 125 are found on this 180,000 kDa subunit of the CA 125 antigen complex.

Antibodies bind to specific epitopes (antigenic determinants) with a single protein species having many different epitopes. Each different subunit of a multimeric complex has its own unique epitopes that differ from those of other subunits associated with the complex. Thus, an antibody directed to one subunit is distinct from an antibody directed to a different subunit.

The OC 125 antibody as taught by the art specifically, reacts with a 180 kDa subunit of the CA 125 antigen complex. The instant antibodies recognize a 40 kDa subunit of the complex. Although the procedure of the present invention used OC 125 to first identify the 40 kDa subunit, this does not mean that OC 125 has a specific reactivity for the 40 kDa subunit. The Examiner's allegations in this regard are unfounded i.e., not scientifically correct. OC 125 binds to the 180 kDa subunit of a larger, stable CA 125 antigen complex.

This entire CA 125 antigen complex can be isolated using OC 125, because the 180 kDa subunit is stably associated with the CA 125 antigen complex. Likewise, the entire complex can be isolated using the antibodies against the 40 kDa subunit. In both cases the same complex is actually isolated, but in neither case do the antibodies recognize the same epitopes. The same result has been achieved by two different means, namely, the use of two antibodies with different specificities for different subunits of the complex.

Although both OC 125 and the instant antibodies are capable of isolating the CA 125 antigen complex, the antibodies are not identical and can readily be distinguished from each other using other immunological techniques readily known to the

skilled artisan. These techniques include radioimmunoassay and immunoblotting. The former measures competitive inhibition between the antibodies (or lack thereof) and the latter shows that OC 125 reacts with a 180 kDa protein of the CA 125 antigen complex (or subunit), while the instant antibodies react with a 40 kDa subunit from the same complex and not vice versa.

Thus, OC 125 and the instant antibodies have different specific reactivities and are, therefore, distinct.

Data generated after the filing date of the present application supports Applicant's position. O'Brien et al. (July, 1986) Am. J. Obstet. Gynecol. 155: 50-55 confirms the heterogeneity and high molecular weight of the CA 125 antigen complex using gel filtration to estimate the size of the complex at >700 kDa and probably in the range of 2000-3000 kDa. It is further demonstrated by an immunoblotting the entire CA 125 antigen complex that OC 125 only detects a 240 kDa and 180 kDa subunit. Likewise, Davis et al. (WO88/03954; June 2, 1988) confirm the size of the CA 125 antigen complex and by immunoblotting show that the specificity of OC 125 is for an about 200 kDa subunit (Figure 5B). In the latter case, Davis et al. analyzed the CA 125 antigen complex from a tumor cell line (OVCA 433) expected to have the 40 kDa subunit and did not report that OC 125 had a specificity reactivity against the 40 kDa subunit.

It is clear from the foregoing that OC 125 does not react with a 40 kDa subunit of the CA 125 antigen complex. Accordingly, the Examiner's rejection of Claims 3 and 4 under 35 U.S.C. § 102(b) is deemed to have been overcome, and withdrawal thereof is respectfully requested.

Further, the law is settled that in order to "anticipate" a claimed invention under 35 U.S.C. § 102(b), a

single prior art reference must disclose each and every element of the claimed invention. Structural Rubber Products Co. v. Park Rubber Co., 749 F.2d 707, 715-716; 223 U.S.P.Q. 1264, 1270 (Fed. Cir. 1984). Both Masuno et al. and Bast et al. fail to disclose an antibody against a subunit (or subspecies) of the CA 125 antigen complex having a molecular weight of about 40 kDa. Consequently, as a matter of law, the Examiner's rejection of Claims 3 and 4 under 35 U.S.C. § 102(b) is overcome and withdrawal is respectfully requested.

In summary, neither Masuno et al. nor Bast et al. anticipate the subject antibodies directed to a 40 kDa subunit of the CA 125 antigen complex.

Even if, pro arguendo, the 40 kDa subunit is a subspecies (or fragment) of the 180 (or 200) kDa subunit, and antibodies against the 40 kDa subunit cross react with the 180 kDa subunit, these antibodies are not the same as OC 125; OC 125 does not detect the 40 kDa subunit on an immunoblot, therefore its antigenic determinants would not be found on the 40 kDa subunit of the instant invention. As the Examiner has acknowledged, it is not obvious from Masuno. et al or Bast et al., that the 40 kDa subunit exists. Further, there is no teaching or disclosure in either reference to analyze the CA 125 antigen complex (i.e., the discovery leading to the present invention) as the Applicant has in order to develop a more specific assay for an ovarian tumor-associated antigen and overcome the limitations of the prior art.

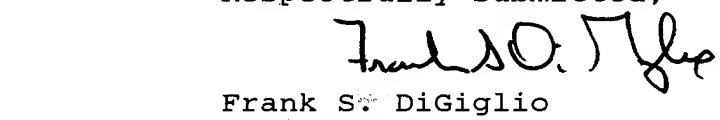
In support of the rejection of Claims 5-10 under 35 U.S.C. § 103, the Examiner has cited the publications to Masuno et al. and Bast et al. in view of either U. S. Patent No. 4,623,621 to Pestka or the WO 85/00663.

The Applicant submits that the preceding comments and remarks with respect to Masuno et al. and Bast et al. are sufficient to overcome the rejection of Claims 5-10 under 35 U.S.C. § 103 in that the secondary references fail to ameliorate the deficiencies of the primary references in any regard; therefore, withdrawal of this rejection is respectfully requested.

The Examiner has inquired with regard to the tissue which was tested for the presence of the 40 kDa subunit of the CA 125 antigen complex in accordance with the specification. Applicants confirm that the normal tissue tested was normal amniotic fluid, upon which tissue the data of the prior art has also been established.

Thus, in view of the foregoing Amendment and Remarks, the present case is deemed to be in condition for allowance which action is earnestly solicited.

Respectfully submitted,



Frank S. DiGiglio
Registration No. 31,346

Scully, Scott, Murphy & Presser
400 Garden City Plaza
Garden City, NY 11530
(516) 742-4343

MLW:pm

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)


(51) International Patent Classification 4 : C12P 21/00, C07K 15/06, A61K 39/00 — A1

(11) International Publication Number: WO 88/ 03954

(43) International Publication Date: 2 June 1988 (02.06.88)

(21) International Application Number: PCT/US87/03052

(22) International Filing Date: 20 November 1987 (20.11.87)

(31) Priority Application Number: 933,784

(32) Priority Date: 24 November 1986 (24.11.86)

(33) Priority Country: US



(71) Applicant: CENTOCOR, INC. [US/US]; 244 Great Valley Parkway, Malvern, PA 19355 (US).

(72) Inventors: DAVIS, Hugh, M. ; 101 North Five Points Road, Apartment F-7, Westchester, PA 19380 (US). KLUG, Thomas, L. ; 208 Brecknock Terrance East, Westchester, PA 19380 (US). ZURAWSKI, Vincent, R., Jr. ; 728 Northgate Road, Westchester, PA 19380 (US).

(74) Agents: DeCONTI, Giulio, A., Jr. et al.; Hamilton, Brook, Smith & Reynolds, Two Militia Drive, Lexington, MA 02173 (US).



(81) Designated States: AT (European patent), BE (European patent), CH (European patent), DE (European patent), FR (European patent), GB (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent).

Published

*With international search report.*

*Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.*


(54) Title: METHOD OF ISOLATING CA 125 ANTIGEN


(57) Abstract

A procedure for isolating CA 125 antigen and an isolated specie of the antigen.


*FOR THE PURPOSES OF INFORMATION ONLY*

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

| | | | | | |
|---|---|---|---|---|---|
| AT | Austria | FR | France | ML | Mali |
| AU | Australia | GA | Gabon | MR | Mauritania |
| BB | Barbados | GB | United Kingdom | MW | Malawi |
| BE | Belgium | HU | Hungary | NL | Netherlands |
| BG | Bulgaria | IT | Italy | NO | Norway |
| BJ | Benin | JP | Japan | RO | Romania |
| BR | Brazil | KP | Democratic People's Republic of Korea | SD | Sudan |
| CF | Central African Republic | KR | Republic of Korea | SE | Sweden |
| CG | Congo | LI | Liechtenstein | SN | Senegal |
| CH | Switzerland | LK | Sri Lanka | SU | Soviet Union |
| CM | Cameroon | LU | Luxembourg | TD | Chad |
| DE | Germany, Federal Republic of | MC | Monaco | TG | Togo |
| DK | Denmark | MG | Madagascar | US | United States of America |
| FI | Finland | | | | |

# METHOD OF ISOLATING CA 125 ANTIGEN

## Background

CA 125 is a tumor-associated antigen expressed on greater than 80% of all non-mucinous ovarian epithelial tumors of serous, endometrioid, clear cell and undifferentiated histologies. Bast, R.C., Jr. et. al., J. Clin. Invest. 68:1331-1337, 1981; Kabawat, S.E. et. al., Am. J. Clin. Pathol., 79:98-104, 1983. The murine monoclonal antibody OC125 which reacts with CA 125 was generated by use of an established human serous cystadenocarcinoma cell line, OVCA 433. Bast, R.C., Jr. et. al., supra. Quantitation of this determinant in serum of patients with ovarian cancer has been made possible by the development of an immunoradiometric assay with OC125. Klug, T.L. et. al., Cancer Res., 44:1048-1053, 1984. The CA 125 antigenic determinant has also been reported to be found in human milk (Hanisch, F.G. et. al., Eur. J. Biochem., 149:323-330, 1985.), in normal cervical mucous (de Bruijn, H.W.A. et. al., Am. J. Obstet. Gynecol., in press) and in the central airway and normal lung tissue (Nouwen, E.J. et. al., Cancer Res., 43:866-876, 1986). In addition, CA 125 activity appears to exist in human seminal plasma .

The CA 125 determinant has been reported to be associated with a mucin-like high molecular weight glycoprotein complex. See e.g., Hanisch, F.G. et. al., Eur. J. Biochem., 149:323-330, 1985; Niloff, J.M. et. al., Am. J. Obstet. Gynecol., 151:981-986.

1986; Bast, R.C. et. al., Ovarian Cancer, pp. 23-35, Boston, MA: Martinus Nihoff, 1985; Masuho, Y. et. al., Cancer Res., 44:2813-2819, 1984; and Bast, R.C., Jr. et. al., Cancer Bull, 37:80-81, 1985. However, the lack of a procedure for isolation of CA125 antigen has impaired analysis of its chemical composition.

Disclosure of the Invention

This invention pertains to a method of isolating the antigen CA 125, to preparations of the isolated antigen and to methods of using the isolated antigen.

The CA 125 antigen can be isolated in high purity as a 200 kDa species from tissue culture medium of ovarian carcinoma cells which "shed" the antigen into the growth medium, (e.g. the human serous cystadenocarcinoma cell line OVCA 433). The CA125 species isolated by the procedure described herein is the same as the species of CA125 found in the serum of patients with non-mucinous ovarian carcinoma, as determined by electrophoretic and immunoblotting analysis.

According to the procedure of this invention, the cell free supernatant is obtained from a culture of human ovarian carcinoma cells. In a first step, protein is precipitated by acid treatment (e.g. perchloric acid, 6M) and the precipitated protein is removed. The acid soluble fraction which contains the CA 125 activity is then neutralized.



CA125 activity in the acid soluble fraction is associated with a high molecular weight complex ( 1,000,000 Da). In the next step, molecular size exclusion chromatography is then used to separate this high molecular weight CA125 species from lower molecular weight components. For example, the acid soluble fraction can be applied to a column of Sepharose™ 4B-CL gel. The Sepharose™ 4B-CL retains molecules of about 60,000-2,000,000 Da. The CA 125 complex is eluted from this column in the void volume.

A chaotropic agent (e.g. Urea, 6M) is used to disrupt the high molecular weight CA 125 complex separated by molecular size exclusion chromatography. The chaotropic agent can be added to the CA 125-containing fraction from the Sepharose™ 4B-CL column. The CA 125 is then separated by a second molecular size exclusion chromatography step. This column is chosen to retain a 200, kDa CA125 species (e.g. Sepharose™ 6Bresin). The chromatography is performed with an elution buffer containing the chaotropic agent and a detergent (e.g. SDS) to stabilize the disrupted CA125. The retained fractions containing CA 125 activity (as determined reactivity with OC125 antibody) are collected as they are eluted from the column. The chaotropic agent is removed from the collected fraction e.g. by dialysis.

In the final step of the isolation procedure, the CA 125 is immunopurified using OC125 antibody. For this purpose, an immunoaffinity column com-

prising immunoactive OC125 coupled to a resin material (e.g. Sepharose 4B) is used.

The CA125 antigen isolated by this procedure has a molecular weight of about 200kDa and a bouyant density of about 1.36 g/ml. The antigen is 24% carbohydrate (by mass). Antibody binding (OC125) activity is heat and protease labile but exoglycosidase and periodate insensitive, indicating that the cognate determinant of OC125 is likely to be proteinaceous.

Isolated CA 125 antigen can be used to raise polyclonal or monoclonal antibody reactive with CA 125. Antibody against OC125 can be used in diagnosis and/or therapy of ovarian carcinoma. e.g. tumor imaging, passive immunotherapy, and immunotoxin therapy. Further, the isolated CA 125 antigen can be used for detection of anti-CA 125 antibody (e.g. by solid phase RIA or ELISA) in the serum, plasma or other biological fluids of patients. The presence of anti-CA 125 antibody in a patient may provide an indication of the existence or recurrence of ovarian carcinoma.

Brief Description of the Drawings

Figure 1 is the elution profile on Sepharose CL-4B column chromatography of the CA 125 antigen isolated from OVCA 433 tissue culture supernatant ( o——o ) and from human serum ( ◇——◇ ).

Figure 2 shows density gradient ultracentrifugation following Sepharose CL-4b column chromatography of the CA 125 antigen isolated from



human serum ( ◇——◇ ), OVCA 433 tissue culture supernatant ( ◆——◆ ), and from human milk ( □——□ ).

Figure 3 shows SDS-PAGE of CA 125 antigen isolated from human milk/4B (200 units/lane) (lane 1), OVCA 433 passage 69/4B (300 units/lane) (lane 3), human ovarian cancer patient sera/4B (100-200 units/lane) (lane 4-7), and from a negative control serum/4B (23 units/lane) (lane 8).

Figure 4 shows conventional SDS:PAGE (3-12% gradient) of CA 125 antigen isolated from OVCA 433 followed by immunoblotting.

Figure 5A shows Sepharose CL-6B elution profile of the CA 125 antigen isolated from OVCA 433. Elution was performed in a SDS-urea-Tris buffer following treatment in 6 M urea at 45°C for 30 min. Fractions were assayed for CA 125 activity with a solid-phase RIA. Figure 5B shows the SDS:PAGE (6%) of pertinent fractions of the Sepharose CL-6B gel filtration column chromatography.

Figure 6 shows density gradient ultracentrifugation of the CA 125 antigen isolated from OVCA 433 following partial purification on a Sepharose CL-4B column ( ◇——◇ ) and following immunoaffinity chromatography purification on an immobilized OC125-Protein A-Sepharose CL-4B column ( o——o ).

Detailed Description of the Invention

The procedure for purifying CA125 generally entails four steps, as indicated below. The procedure can be applied to isolate CA125 from

tissue culture media in which ovarian carcinoma cells have been grown. The cells, of course, must be ovarian carcinoma cells which express the antigen and "shed" (release) the antigen into the growth medium. The procedure may also be used to isolate the antigen from biological fluids such as serum or ascites. However, the minute quantities found in these fluids generally make them an impracticable source of the antigen for purification.

The preferred ovarian carcinoma cell line is the OVCA 433 cell line described by Bast, R.J., Jr. et al., supra. Other cell lines which can be used are the ovarian tumor cell lines NIH:OVCAR-3 (ATCC #HTB161), SK-OV-3 (ATCC # HTB77), CAOV-3 (ATCC # HTB75), and CAOV-4 (ATCC # HTB76). When grown in a conventional tissue medium, these cell lines release CA125 antigen into the medium. The released antigen can then be isolated from the medium by the four step procedure.

1. Acid Precipitation

Cell-free supernatants are subject to acid precipitation. The preferred acid is perchloric acid at 0.6M final concentration. Precipitated protein is removed and the acid soluble fraction which contains the CA125 activity is neutralized (e.g. with KOH). The acid soluble fraction can then be dialyzed against distilled water and concentrated (e.g. 20X original supernatant volume).

2. Molecular Size Exclusion Chromatography

The acid soluble fraction is submitted to molecular size exclusion chromatography to separate the high molecular weight CA125 complex from lower



molecular weight components. A preferred resin is Sepharose CL-4B resin which retains molecules in the 60 kD to 2,000 kD range. The CA125 antigen is eluted from this column in the void volume. The antigen can be applied and eluted in phosphate buffered saline (PBS)

3. Treatment with Chaotropic Agent and Molecular Size Exclusion Chromatography

The fraction containing CA125 activity is treated with a chaotropic agent. The chaotropic agent disrupts the high molecular weight CA125 complex. Urea is preferred, but quanidine-HCl may also be used. Urea treatment is followed by chromatography with a resin that retains molecules in the 200 kD range. A preferred resin is Sepharose CL-6B. The chromatography on Sepharose CL-6B is done with a buffer containing the chaotropic agent (e.g. 6M Urea) and a detergent (e.g. 1% SDS). The eluted fraction can be monitored for CA125 activity by CA125 RIA.

4. Affinity Purification

The CA125 antigen is purified by immunoaffinity chromatography. OC125 is bound to a solid phase (e.g. Protein A-Sepharose CL-4B resin) and the antigen containing fraction from the prior gel filtration step is passed over the resin under conditions which allow the antigen to bind specifically to the solid phase. The antigen is then eluted with an appropriate eluant such as diethylamine. The preferred immunoaffinity column is prepared essentially by the methods of Schneider et. al., infra.

OC125 is covalently coupled to Protein A-Sepharose via the coupling agent dimethylpimelimidate, a coupling which does not interfer with the activity of the OC125 antibody. Bound antigen is eluted with diethylamine.

In the preferred embodiment, CA 125 antigen is purified from OVCA 433 cell culture supernatants using the procedure described below.

Purification Step 1. Cell culture supernatants from OVCA 433 culture are collected from confluent monolayer cultures. Supernatants are concentrated 10 fold and made 0.6 molar in perchloric acid. Precipitated protein is removed. The acid soluble fraction is neutralized, and then dialyzed against distilled water. The CA 125 reactivity is found in the soluble fraction (95%), while 80% of the protein is removed.

Purification Step 2. The perchloric acid soluble fraction is concentrated and fractionated by gel filtration chromatography using a 3.2 X 85 cm column of Sepharose$^{TM}$ 4B-CL equilibrated in phosphate buffered saline (PBS). CA 125 activity and $A_{280}$ are determined for each fraction. The majority of CA 125 activity is eluted at the void volume (Vo) and a smaller peak which elutes later. This profile indicates the presence of a high molecular weight component (>1000 kDa) and a smaller component (200 to 400kDa). The Vo fractions contain about 85% of the initial reactivity.

Purification Step 3. The Vo fraction from the Sepharose 4B-CL column is made 6M in urea and then applied to a 1.2 X 95 cm Sepharose 6B-Cl column in 0.1% SDS, 6M urea, 50 mM Tris HCl, pH 8.0. Fractions are collected and assayed for CA 125



reactivity. The CA125 activity is eluted in two peaks: Peak 1, a minor, high molecular weight component (>1000 kDa) at the void volumne and Peak 2, a major, lower molecular weight component (200 to 400 kDa).

Purification Step 4. The material is peak 2 is further purified by immunoaffinity chromatography. An affinity column is prepared by covalently coupling OC125 antibody to a Protein A-Sepharose 4B-CL column according to the method of Schneider et. al. The pooled Peak 2, still in 6M urea, 0.1% SDS, is passed over the immunoaffinity column three times. After washing, the antigen is eluted with 50mM diethylamine (DEA) pH 11.5. The eluate is immediately neutralized by collection into a neutralizing buffer, and is then dialyzed against distilled water.

The antigenic activity of CA 125 at each step of this purification has been evaluated using Western blots. Most of the reactivity from Purification Step 1 (PCA extraction) has a very high molecular weight (>1000 kDa), and little or no reactivity is found in the low molecular weight region (<1000 kDa). Analysis of the peak 2 from Purification Step 3 indicates that most of the reactivity is in the 200-400 kDa region. This suggests that the 1000 kDa antigen dissociates into a smaller component. Finally, in Purification Step 4, the antigen eluted from the immunoaffinity column has the 200-400 kDa component with no detectable 1000 kDa reactivity.

To demonstrate that the 200-400 kDa components noted after Steps 3 and 4 were formed by dissociation of the >1000 kDa material, Western blots were performed on a PCA extract, with and without treatment with 6M urea. The untreated PCA extract had a major component of 1000 kDa and a minor 200-400 kDa component. After treatment with 6M urea (45° for 30 minutes), most of the reactivity was found in the 200-400 kDa region.

The isolation procedure of this invention can give a 3900-fold purification relative to starting supernatant material (as determined by activity in units/mg protein in starting material versus final material). The CA125 antigen species isolated by this procedure is characterized by the following:

It has a molecular weight of about 200 kDa. It is comprised of 24% carbohydrate. The carbohydrate composition is sialic acid, fucose, mannose galactose, N-acetyl-glucosamine, and N-acetyl galactosamine in the ratio 3.6/0.4/3.0/6.6/5.8/2.2.

The region of the OC125 determinant appears to be proteinaecous (See exemplification below).

The isolated immunoreactive 200 KDa species of CA 125 can be used as an immunogen preparation to raise anti-CA 125 antibody. For example, monoclonal anti-CA125 antibodies can be produced by standard techniques of Kohler and Milstein. A mouse is immunized with the isolated CA125. Spleen cells are harvested and fused with myeloma cells. Resulting hybridomas can be selected for anti-CA 125 antibody production on the basis of reactivity with isolated CA 125 antigen.



Antibody against CA 125 is useful for diagnosis and therapy of ovarian carcinoma. For example, the antibody can be used in diagnostic tests such as RIAs and ELISAs for the presence of CA 125 in biological fluids. Such antibody can be used in immunohistochemical techniques for identification of tumor techniques for identification of ovarian carcinoma cells. The antibodies may also be used for in vivo imaging of ovarian cancer and for immunotherapy of ovarian cancer e.g. passive immuno therapy or immunotoxin therapy.

The isolated CA 125 can also be used to provide an immunoadsorbent for detection of anti CA125 antibody in the blood. The presence of CA125 antibody may provide an indication of ovarian carcinoma in a patient.

The invention is illustrated further by the following exemplification.

## Exemplification

Materials and Methods

### Materials

The murine monoclonal antibody OC125, produced by hybridomas grown in pristane primed BALB/c mice (Bast, R.C. et. al. J. Clin. Invest., 68:1331-1337, 1981), was isolated by protein A chromatography (Kabawat, S.E. et. al. Am. J. Clin. Pathol., 79:98-104, 1983). Serum samples were obtained from women with advanced epithelial ovarian cancer (stage III and IV). Human milk was obtained from a healthy 7-month post partum female. The exoglycosidases and

proteases were purchased from Calbiochem, Los Angeles, CA (pronase), and from Sigma, St. Louis, MO (chymotrypsin, trypsin, chondroitinase ABC, $\alpha$-and $\beta$-galactosidase, $\alpha$-fucosidase, hexaminidase, and neuraminidase). Monoclonal antibody 1116NS 19-9 (Koprowski, H. et. al. Somat. Cell Genet:5(6):-957-972, 1979; U.S. Patent 4,349,528) was obtained from Dr. Zenon Steplewski, Wistar Institute, Philadelphia, PA. Polyclonal anti-CEA antibody was obtained from Abbott Laboratories, North Chicago, IL. Sepharose CL-4B and CL-6B and Protein A-Sepharose CL-4B were purchased from Pharmacia, Piscataway, NJ. Electrophoresis reagents were purchased from Bio-Rad, Rockville Centre, NY. SeaKem LE agarose was purchased from FMC Corp., Rockland, ME. Fish gelatin was obtained from Norland Products Inc., New Brunswick, NJ. All other reagents were of the highest purity commercially available.

Solid-Phase Radioimmunoassays

The simultaneous "sandwich" immunoradiometric assay (IRMA) was used to measure CA 125 activity (Klug, T.L. et. al. Cancer Res., 44:1048-1053, 1984.) and CA 19-9 activity (Ritts, R.E. et. al. Int. J. Cancer, 33:339-445, 1984). In the CA 125 IRMA, [$^{125}$I]-OC125 (100 ul, 1x10$^5$ cpm) was incubated (20 h, 23°C) with polystyrene-immobilized OC125 and sample (100 ul). The beads were washed (3X) and counted in a gamma counter. Assay kits were manufactured at Centocor, Malvern, PA.

The plate assay was performed using 96 well polyvinyl chloride microtiter plates (Dynatech).



The OVCA 433/PCA/4B (see "Isolation of CA 125 from OVCA 433 tissue culture supernatants") fraction was used to coat the wells (100 ul, 500 units/well). Following the binding of the antigen to the plates (18 h, 4°C), the wells were incubated for 1 h with phosphate buffered saline (PBS) containing 5% (w/v) bovine serum albumin. After the incubation period, the wells were emptied and washed (2X) with PBS. [$^{125}$I]-OC125 (20 ul, 2x10$^4$ cpm) was then incubated with the immobilized antigen (4 h, 23°C). The wells were subsequently washed (3X) with PBS, cut, and counted in a gamma counter.

As the CA 125 IRMA only detects polyvalent antigens, an inhibition assay was developed to quantitate both mono- and multi-valent antigens. The inhibition assay was performed similarly to the plate assay described above, the only difference being that [$^{125}$I]-OC125 (20 ul, 2x10$^4$ cpm) was incubated simultaneously (30 ul, 4 h, 23°C) with various antigen preparations which might inhibit binding of radiolabeled OC125 to the plate. The wells were washed (3X), cut, and counted in a gamma counter. The radioiodinated OC125 used in both the plate and inhibition assays was obtained from Centocor RIA kits.

SDS:Polyacrylamide Gel Electrophoresis

Conventional SDS:PAGE was performed essentially according to the method of Laemmli (Laemmli, U.K. Nature 227:680-685, 1970.). The sample buffer did not contain sulfhydryl reducing agents or SDS and was not heated, as the CA 125 antigen was

inactivated by these conditions. Some experiments required a polyacrylamide-agarose composite gel for separation of sample components as the CA 125 antigen did not penetrate a conventional 3% (w/v) polyacrylamide gel.

Typically, the composite gels were prepared with 2.5% polyacrylamide and 1.0% agarose. The solutions were heated to 65°C at which time the ammonium persulfate was added. The prewarmed solutions were then immediately poured into the gel apparatus which had been equilibrated at 37°C and the entire apparatus was then cooled at 4°C until the agarose solidified. After overlaying a 2.5% polyacrylamide-1.0% agarose stacking gel at room temperature the samples (300 units/lane) were applied in 10 M urea sample buffer which did not contain sulfhydryl reducing agents or SDS and was not heated. The electrophoresis was performed at 4°C. All buffers used in the preparation and running of the composite gels were also those of Laemmli (See supra.).

Immunoblotting

After electrophoresis the proteins were electrophoretically transferred to nitrocellulose (Towbin, et al., Proc. Natl. Acad. Sci. 76:4350-4354, 1979)), immunoblotted with radiolabeled OC125, and autoradiographed. Each immunoblot contained at least one negative antigen control lane. The elctrophoretic transfer was performed at 100 mA overnight. Immunoblotting was accomplished by overlaying the nitrocellulose with



radioiodinated OC125 (2 ml, $2x10^6$ cpm) in fish gelatin buffer (1% fish gelatin, 50 mM citrate, pH 6.0, 0.05% NP-40) for 6 h. The nitrocellulose sheet was then autoradiographed by exposure to x-ray film with the aid of a Cronex Quanta III fluor screen (Dupont) for 18 h at -80°C.

Fractionation of Human Serum and Human Mild

Whole serum was allowed to clot for 1 h and then centrifuged (3,000 x g, 10 min). A portion (2 ml) of the supernatant was fractionated on a 1.2 x 47 cm Sepharose CL-4B column (human serum/4B) equilibrated in PBS. Fractions (1 ml) containing CA 125 activity, as determined by the CA 125 RIA, were pooled and concentrated. Human milk was defatted by centrifugation (3,000 x g, 1 h) at 10°C. The supernatant was further purified by column chromatography as described above for serum (human milk/4B).

Preparation of CA 125 Antigen Concentrate from OVCA 433 Tissue Culture Supernatant

OVCA 433 human ovarian carcinoma cells were grown in Minimum Essential Medium Eagle supplemented with 2 mM glutamine, 1 mM pyruvate, 1% non-essential amino acids, and 10% heat-inactivated fetal calf serum. T-150 flasks (Costar) were seeded with $1x10^6$ cells. Growth was permitted to continue until cells reached confluence at which time the medium was removed. Fresh medium was added and collected at 5-7 day intervals, for a total of 10-12 weeks. OVCA 433 cells appeared to produce the maximum amount of CA 125 antigen in $G_o$ growth phase. The concentra-

tion of CA 125 antigen produced under these conditions was approximately 1,000 units/ml. Pooled cell supernatants were centrifuged at 10,000 x g, filtered through a Sartorius 0.2 micron pore size cascade filter capsule, and concentrated to one-tenth the original volume with an Amicon DC-2 hollow fiber apparatus and filter cartidge (HP 100-200) with a molecular weight cutoff of 100 kDa. The concentrates were stored frozen at -20°C under which conditions the CA 125 activity was stable for at least 12 months.

Isolation of CA 125 from OVCA 433 Tissue Culture Supernatant

The spent tissue culture 10X concentrate of the OVCA 433 cell supernatant was first subjected to perchloric acid (PCA, 0.6 M final concentration) precipitation (Krupey, J. et. al. J. Exp. Med. 128:387-398, 1968). The CA 125 activity remained in the PCA soluble fraction and was completely conserved. The acid soluble fraction was neutralized with potassium hydroxide (1.2 M), dialyzed against distilled water (24 h, 4°C), and concentrated to 20X the original supernatant volume. This sample is referred to as OVCA 433/PCA. The OVCA 433/PCA sample (35 ml) was applied to a Sepharose CL-4B column (3.2 x 70 cm) equilibrated in PBS. The fractions (7 ml) which contained CA 125 activity as determined by the CA 125 RIA were pooled and concentrated. This fraction is referred to as OVCA 433/PCA/4B and is used in all experiments except as indicated.



Further fractionation involved treatment of the OVCA 433/PCA/4B fraction with urea (6 M, 30 min, 45°C) and subsequent chromatograph on a Sepharose CL-6B column equilibrated in Tris-urea-SDS (50 mM Tris, 6 M urea, 0.1% SDS, pH 8.0). Final fractionation was accomplished by immunoaffinity chromatography on an OC125-Protein A-Sepharose CL-4B column. The monoclonal antibody OC125 was covalently bound to the Protein A-Sepharose CL-4B column, washed and coupled essentially according to the method of Schneider et al., J. Biol. Chem. 257:10766-10769, 1982. Minor modifications included the substitution of citrate buffer (0.05 M, pH 6.0) for Tris-HCl, and taurodeoxycholate (TDC) for deoxycholate (DDC). Repeated passes (3X) over the affinity column of the CA 125 reactive lower molecular weight fraction from the Sepharose CL-4B column in 0.1% SDS and 6 M urea gave greater than 80% binding of the CA 125 activity. Elution of the CA 125 antigen from the column was accomplished with the use of diethylamine (DEA) (50 mM, pH 11.3). This affinity purified antigen is referred to as OVCA 433/4B/DEA.

Density Gradient Ultracentrifugation

Ultracentrifugation of the CA 125 antigen isolated either from human serum, human milk, or from the OVCA 433 tissue culture supernatant after chromatograpy on Sepharose CL-4B was performed in a cesium chloride isopycnic density gradient in PBS (2.276 g of CsCl dissolved in 3.414 ml of PBS). The buoyant density of B-galactosidase was determined as

a standard. Fractions (0.2 ml) were assayed for B-galactosidase activity following equilibrium by the method of Miller (Experiments in Molecular Genetics, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1972). Gradients were formed by ultracentrifugation in a Beckman SW50.1 rotor (33,000 rpm, 68 h, 10°C) under conditions which have been described (See, Magnani, J.L. et. al., Cancer Res; 43:4589-5492, 1983). Fractions (0.2 ml) were collected and assayed for activity using the CA 125 RIA described above. The density of each fraction was determined by weighing a known volure.

Chemical Treatments

Periodate oxidation of the CA 125 antigen was accomplished with 0, 0.1, 1.0, 10.0 and 100 mM periodate in acetate buffer (pH 4.5, 50 mM, 4°C) in the dark according to Stahl et al., Proc. Natl. Acad. Sci; 73:4045-4049, 1976. Reduction and alkylation were performed according to methods described elsewhere. See, Glazer, A.N. et. al., Chemical Modifications of Proteins. In: T.S. Work and E. Wbrk/Eds) Laboratory Techniques in Biochemistry and Molecular Biology, p. 104, New York:Elsevier Publishing Co., 1975. Reduction was accomplished with DTT (10 mM, 50 mM Tris, pH 8.1, 4 h, 45°C) either in the presence or absence of guanidine-HCl (6 M). Alkylation was performed with iodoacetic acid (20 mM, 30 min) after the samples had been cooled to room temperature. The samples were immediately dialyzed (4°C, 18 h) against distilled water.



Exoglycosidase Treatments

Exoglycosidase digestions were performed in acetate buffer (.01 M, pH 4.5, 48 h, 37°C). Unit values of the exoglycosidases were chosen in order to ensure complete digestion of the oligosaccharide residues within an appropriate time frame. All exoglycosidase digestions were performed under conditions whereby the appropriate substrates were shown to be completely hydrolyzed as detected by thin layer chromatography. CA 125 activity following treatment was measured both by the CA 125 RIA and by the plate assay as previously described.

Exhaustive Protease Digestion

The various protease digestions were performed in Tris-HCl buffer (0.2 M, pH 8.0, 10 mM calcium chloride). The proteases trypsin, chymotrypsin, and pronase (2% w/v, 50 ul) were added to wells containing antigen and allowed to incubate (48 h, 37°C). Protease digestions were performed under conditions which caused hydrolysis of albumin as detected by thin layer chromatography. Samples were assayed for CA 125 activity by both the CA 125 RIA and the plate assay as described above.

Amino Acid Analysis

Samples of OVCA 433/4B/DEA were dissolved in 6N HCl containing 0.1% phenol, sealed under vacuum, and hydrolyzed for 24 h at 110°C. Amino acids were derivatized with phenylisothiocyanate (PITC) and derivatized PTC-amino acids separated and quantitated by HPLC using the Waters PICO-TAG column and

elution conditions See Bidlingmeyer, B.A. et. al. J. Chromatogr., 336:93-104 (1984).

Carbohydrate Compostion

Samples of the same lot of OVCA 433/4B/DEA that had been subjected to amino acid quantitation were subjected to carbohydrate compositional analysis as described by Yang and Hakomori, J. Biol. Chem. 246:1192-1200, 1971. The samples were subjected to acetolysis, followed by hydrolysis and reduction. The resultant alditols were per-O-acetylated with acetic anhydride. Quantitation of sialic acid was accomplished by trimethylsilyl derivatization (TMS). Laine, R.A. et. al., Meth. Enzymol. 28:159-167, 1972. Both the alditol acetate and the TMS-methyl glycosides were separated by a Hewlett Packard 5790 gas chromatograph and identified by a Hewlett Packard 5790 mass selective detector (MSD).

Results

Physical and Immunological Characteristics of the CA 125 Antigen

The antigen isolated from OVCA 433 and from human ovarian cancer patient serum by perchloric acid precipitation elutes primarily in the void volume of a Sepharose CL-4B column (Figure 1). In addition, a smaller peak of CA 125 activity elutes from the column later indicating a much lower molecular weight. These peaks of CA 125 activity correspond to molecular masses of greater than 1,000 and about 200-400 kDa. The CA 125 antigen elution



pattern of human milk is similar to that shown for OVCA 433 and human cancer patient serum.

In an effort to compare the physical characteristics of the antigen isolated from OVCA 433 cell supernatants, ovarian cancer patient serum, and human milk, a buoyant density was determined for each (Figure 2). The average buoyant density of the antigen isolated from OVCA 433 after passage over a Sepharose CL-4B column (OVCA 433/4B) is approximately 1.42 g/ml whereas the buoyant densities of the patient serum/4B and the milk/4B are 1.46 and 1.39 g/ml, respectively. As a standard, the buoyant density of >-galactosidase was determined and found to be 1.32 g/ml. This agrees well with the published value of 1.316 g/ml Costini, N.V. et. al., J. Biol. Chem; 254:11242-11246, 1979. Figure 3 compares the electrophoretic mobility of immunoreactive species from OVCA 433/4B, human milk/4B, and ovarian cancer serum/4B on a composite 2.5% polyacrylamide/1.0% agarose gel. The samples were applied in 10 M urea sample buffer which did not contain DTT or SDS and was not heated. The immunoblotted OC125 reactive antigen from each of the sources is present as high molecular mass complexes of between 200 and 1,000 kDa with similar electrophoretic profiles. This data, which suggests multiple aggregated states of the CA 125 antigenic complex, correlates well with the Sepharose CL-4B elution profile shown in Figure 1. Both experiments indicate that antigen exists as a high molecular mass species of greater than 1,000

kDa and lower molecular mass species of approximately 200-600 kDa.

When the OVCA 433/PCA/4B fraction is subjected to SDS:PAGE electrophoresis using a 3-12% polyacrylamide gradient gel followed by immunoblotting (Figure 4), the lane which is reactive with radioiodinated monoclonal antibody OC125 give rise to a band of greater than 1,000 kDa molecular mass and a lower molecular mass band of approximately 400 kDa. The sample buffer used contained only 10% glycerol, 0.08 M Tris, pH 6.8, and bromophenol blue. After overlaying the adjacent lane with radioiodinated monoclonal antibody 19-9, which recognizes the sialylated lacto-N-fucopentaose II carbohydrate determinant, only the higher molecular mass band is observed. The lane which is overlaid with radioiodinated anti-CEA does not shown any immunoreactivity. Furthermore, Western blots using monoclonal antibody 19-9 as an overlay with the OC125 affinity purified CA 125 antigen (OVCA 433/4B/DEA) fraction does not give rise to any bands. Also, there is no CA 19-9 activity present when measured by the CA 19-9 RIA (data not shown). This result clearly demonstrates that the antigenic determinants CA 125 and CA 19-9 are located on the same high molecular mass glycoprotein complex, but the CA 125 and CA 19-9 determinants are not present on the same glycoprotein molecule.

The results of Sepharose CL-4B column chromatography and of SDS:PAGE analysis suggests that the lower molecular weight material was probably derived



from the higher molecular weight species. Attempts to disaggregate the high molecular weight material with both ionic (SDS) and non-ionic (NP-40) detergents proved futile. However, treatment of the pooled and concentrated void volume fraction of the Sepharose CL-4B column of OVCA 433/PCA with 6 M urea for 30 min at 45°C, followed by column chromatography on Sepharose CL-6B in 0.1% SDS and 6 M urea yields two peaks, as shown in Figure 5. Following this step, the majority (80%) of the CA 125 activity is found associated with a much lower molecular mass peak of approximately 200 kDa. This is verified by electrophoresis and immunoblotting of fractions from the Sepharose CL-6B column chromatography (Fig. 5). Some of the antigen still remains in the high molecular mass aggregated form.

Immunoaffinity Purification of the CA 125 Antigen from OVCA 433 Cells. Sepharose CL-4B column chromatography followed by treatment with 6 M urea and heat with subsequent column chromatography on Sepharose CL-6B in the presence of 6 M urea and 0.1% SDS (Fig. 5) results in a 1,400-fold purification of CA 125 antigen from OVCA 433 supernatants (data not shown). This preparation has a specific activity of 117 units CA 125/ug of protein. The specific activity is determined by measuring the CA 125 activity using the Centocor CA 125 RIA kit and determining the amount of protein by amino acid analysis on this same lot of purified CA 125 antigen. Final fractionation of the antigen is

accomplished by immunoaffinity on an immobilized OC125-Protein A-Sepharose Cl-4B column. The antigen which eluted from the column with diethylamine (DEA) has a specific activity of 317 units CA 125/ug of protein.

Samples of antigen eluting from a Sepharose CL-4B column and from an OC125 immunoaffinity column were subjected to density gradient ultracentrifugation. This procedure reveals different average bouyant densities for the two antigen preparations (Fig. 6). The more highly purified DEA eluate has a bouyant density of approximately 1.36 g/ml whereas the bouyant density of the OVCA 433/4B is approximately 1.42 g/ml. This suggests that the less pure antigen is associated with more highly glycosylated proteins which would result in the polydisperse nature of the bouyant density profile as well as the higher average bouyant density observed.

Carboyhydrate Composition of the CA 125 Antigen Isolated by Affinity Chromatography. Preliminary carbohydrate composition of OVCA 433/4B/DEA reveals that sialic acid, fucose, mannose, galactose, N-acetyl glucosamine, and N-acetyl galactosamine are present in the ratio 3.6:0.40:3.0:6.6:5.8:2.2, respectively (data not shown). This data suggests that there are both N- and O-linked oligosaccharides present. In addition, this immunopurified CA 125 antigen is found to contain 24% carboyhydrate, by mass, in close agreement with that calculated from its bouyant density of 1.36 g/ml. Therefore, the CA



125 antigen is not a typical mucin and does not have a significant amount, if any, of lipid associated with it.

Nature of the CA 125 Determinant. The nature of the CA 125 determinant was investigated using a number of chemical and physical treatments, as well as exhaustive exoglycosidase and protease digestions of the antigen. Periodate oxidation (Table I) of the CA 125 immunoreactive antigen isolated from OVCA 433/4B and from human milk/4B has no effect on activity at periodate concentrations and reaction times that totally destroyed activity of the CA 19-9 carbohydrate determinant, the sialylated lacto-N-fucopentaose II. In fact, at the lowest periodate concentrations which destroyed CA 19-9 activity (0.1 mM) there actually appears to be an increase in CA 125 activity. Only at very high concentrations of periodate (100 mM) or at very long reaction times (24 h) is there a significant decrease in CA 125 activity, which is likely due to non-specific oxidation of the antigen protein backbone.

TABLE I

EFFECT OF PERIODATE OXIDATION ON CA 125 ACTIVITY AT VARIOUS CONCENTRATIONS AND REACTION TIMES.

| SAMPLE | PERIODATE CONCENTRATION (mM) | CA 125 Activity (% Remaining) TIME (h) 0 | 3 | 6 | 24 |
|---|---|---|---|---|---|
| OVCA 433/ PCA/48 | 0 | 100 | 99 | 102 | 102 |
| | 0.1 | 100 | 110 | 115 | 92 |
| | 1.0 | 100 | 134 | 144 | 121 |
| | 10.0 | 100 | 151 | 135 | 119 |
| | 100.0 | 100 | 72 | 48 | 40 |
| Human milk/ 48 | 0 | 100 | 101 | 103 | 98 |
| | 0.1 | 100 | 103 | 93 | 97 |
| | 1.0 | 100 | 107 | 110 | 84 |
| | 10.0 | 100 | 111 | 69 | 50 |
| | 100.0 | 100 | 7 | 5 | 5 |
| Positive Control 19-9 cGP | 0 | 100 | 100 | 97 | 99 |
| | 0.1 | 100 | 16 | 11 | 5 |
| | 1.0 | 100 | 10 | 5 | 5 |



Chemical and physical treatments (Table II) which denature most proteins, that is, reduction and alkylation in 6 M guanidine-HCl, 8 M urea, and boiling all reduce the CA 125 immunoreactivity substantially. Reduction alone, however, does not seem to affect CA 125 immunoreactivity. Thus, the decrease in activity observed with either reduction of alkylation in the presence of guanidine-HCl is mainly the result of guanidine-HCl acting on the antigen. There is almost complete loss of activity with reduction and alkylation in the presence of guanidine-HCl. In addition, neither the anionic detergent SDS nor the non-ionic detergent NP-40 affects the CA 125 immunoreactivity.

TABLE II

THE EFFECT OF CHEMICAL TREATMENTS ON CA 125 ACTIVITY

| Treatment | CA 125 Activity (% Remaining) | | |
|---|---|---|---|
| | Plate Assay | Inhibition Assay | IRMA (Sandwich) |
| Control | 100 | 100 | 100 |
| Guanidine-HCl(6M, 45° 4h) | ND | 20 | 49 |
| Reduction (10 mM DTT, 4h, 45°C) | ND | 73 | 98 |
| Reduction in guanidine-HCl | ND | 40 | 20 |
| Alkylation (20mM iodoacetic acid, 30 min, 23°C) | ND | 31 | 82 |
| Alkylation in guanidine-HCl | ND | 40 | 29 |
| Reduction and alkylation | ND | 53 | 51 |
| Reduction and alkylation in guanidine-HCl | 5 | 12 | 5-7 |
| Urea (8M, 24h, 4°C) | 100 | 100 | 100 |
| Urea (8M, 24h, 45°C) | 15 | 10 | 0 |
| Heat (100°C, 20 min.) | 0 | 0 | 0 |
| SDS (2%) | 100 | ND | 100 |
| NP-40 (10%) | 100 | ND | 100 |

ND designates assay was not done.





Various combinations of exoglycosidase treatments were performed on the CA 125 antigen (Table III). The solid-phase IRMA indicates only slight losses of CA 125 immunoreactivity either with $\alpha$-galactosidase and/or $\beta$-galactosidase treatments. On the other hand, no loss of immunoreactivity is demonstrated using the plate assay. In fact, there is an increase in the ability of the immobilized antigen to bind radiolabeled OC125 antibody following most of the exoglycosidase treatments. This result corroborates that obtained with periodate oxidation, that is, removal of terminal carbohydrate moieties may actually increase access of OC125 to the CA 125 determinant.

Finally, exhaustive protease digestion with pronase, trypsin, or chymotrypsin causes complete loss of antigenic activity as measured with either the IRMA or the plate assays (Table III).

TABLE III

THE EFFECT OF ENZYMATIC DIGESTION ON CA 125 ANTIGEN ACTIVITY ISOLATED FROM OVCA 433

| Exoglycosidase Treatment | CA 125 Activity (% Remaining) IRMA | Plate Assay |
|---|---|---|
| Control | 100 | 100 |
| Neuraminidase (N) | 96 | 126 |
| N + α-Fucosidase (F) | 106 | 128 |
| N + F + β-Galactosidase(βG) | 96 | 129 |
| N + F + βG + Hexosaminidase | 109 | 123 |
| α-Galactosidase | 94 | 117 |
| α + β-galactosidase | 88 | 116 |
| Chondroitinase ABC | 93 | 94 |

| Exhaustive Protease Treatment | IRMA | Plate Assay |
|---|---|---|
| Pronase | 0 | 0 |
| Trypsin | 0 | 0 |
| Chymotrypsin | 0 | 0 |



Discussion

The murine monoclonal antibody OC125 recognizes a human ovarian carcinoma-associated antigenic determinant (CA 125). We have isolated glycoprotein complexes from the ovarian cancer cell line OVCA 433, human serum, and human milk all of which express CA 125 determinant activity. In addition, we have evidence of CA 125 activity in seminal plasma which is in contrast to the observations of de Bruijn et. al. supra. Chemical treatment and chromatography of the high molecular weight complex isolated from OVCA 433 cell supernatants gave rise to a 200 kDa immunoreactive species. It is possible, however, that the actual protein which expresed the antigenic determinant may be of still lower molecular weight. Further attempts to isolate a lower molecular weight immunoreactive species have thus far proven ineffective. Moreover, the isolation scheme described here does not give rise to a completely homogenous and pure species.

The antigen expressing the CA 125 determinant isolated from several sources exists as a high molecular weight glycoprotein complex with an average bouyant density of between 1.36 and 1.46 g/ml. Moreover, these average densities indicated that each of the antigens isolated from three sources may have had a slightly different protein and carbohydrate composition. If a mucin is defined as a high molecular weight glycoprotein composed of 50% or more carbohydrate with a majority of O-linked

oligosaccharides containing little ore no N-linked chains, then the CA 125 antigen is not a typical mucin. This conclusion is based on the CA 125 carbohydrate composition of 24%, the high amount of mannose present, the majority of N-linked oligosaccharides, and the CA 125 antigen bouyant density. The average bouyant density of unglycosylated protein is between 1.25 and 1.35 g/ml, while the average bouyant density of mucins is approximately 1.50 g/ml. This finding is in contrast to that reported for other epithelial tumor-associated antigens recognized by monoclonal antibodies such as 19-9 (Magnani, J.L. et. al., J. Biol. Chem; 257:14365-14369, 1982), B72.3 (Johnson, V.G. et. al., Cancer Res.; 45:850-857, 1986), DU-PAN-2 (Lan, M.S. et. al., Cancer Res; 45:305-310, 1985), and F36/22 (Croghan, G.A. et. al.; Cancer Res. 43:4980-5988, 1983), all of which have been classified as high molecular weight mucin-like glycoproteins based on their higher bouyant densities.

The higher molecular weight antigen complex isolated from the supernatant of OVCA 433 was reactive with the monoclonal antibody 19-9 (Magnani et. al. supra,), suggesting that the CA 19-9 determinant is present on this complex. However, we have clearly shown by electrophoresis and immunoblotting that the CA 19-9 and the CA 125 determinants were not located on the same glycoprotein since the OC125 immunoaffinity purified CA 125 antigen showed no reactivity with the monoclonal antibody 19-9. This



observation is contrary to that suggested by Hanisch et. al. supra who had isolated both CA 19-9 and CA 125 activity from human milk.

Chemical and physical treatments of CA 125 antigen were undertaken to better understand the nature of the antigenic determinant recognized by the monoclonal antibody OC125. Periodate oxidation of CA 125 reduced the immunoreactivity only at high concentrations of periodate or with prolonged reaction times. In fact, the activity of the antigen actually increased at concentrations and reaction times which caused total loss of immunoreactivity of the CA 19-9 determinant. Non-specific oxidation of the protein backbone probably caused the loss of CA 125 activity at higher concentrations of periodate. During CA 125 antigen purification, there was a loss of 82% of the original activity following urea and heat treatment. This apparent loss in activity was most likely due to breakdown of antigen complex to a less aggregated form or to partial denaturation of the antigen. A lower aggregated state may lead to a lower unit value as the CA 125 RIA is sensitive to CA 125 antigen valency, i.e. the number of OC125 binding sites per antigen molecule.

The observations of Hanisch et. al. supra which suggested that the CA 125 determinant is carbohydrate in nature were based on two criteria; its sensitivity to periodate oxidation (at a concentration of 100 mM and a reaction time of (18 h),

and its loss of activity under conditions which would selectively cleave N-acetylneuraminic acid (pH 3.3, 100°C). Their results also indicated that neuraminidase treatment alone caused only slight reduction of immunoreactivity even though approximately 97% of the mucin-linked sialic acid awas cleaved. Our results clearly show that concentrations of periodate sufficient to oxidize carbohydrates do not affect CA 125 activity. It is not surprising, therefore, that pH 3.3 at 100°C destroyed CA 125 antigenic activity. In addition, greater than 95% of the activity was lost upon reduction and alkylation treatment in the presence of guanidine-HCl. Lastly, exoglycosidase treatments actually caused an increase in CA 125 activity while antigen activity was completely eradicated with exhaustive protease digestion. These data strongly suggest that the CA 125 determinant is proteinaceous in nature, or at the very least, is protein associated with carbohydrate in a conformationally dependent epitope. This may explain the similarity of the antigen isolated from the various sources such as human serum, OVCA 433, and human milk. A peptide determinant would be expected to be more highly conserved than a carbohydrate determinant, that is, a protein sequence is more likely to be associated with a single unique protein, whereas a carbohydrate structure may exist on several different proteins. These results may not be completely unique as the nature of the tumor associated glycoprotein epitope (TAG-72) recognized by the



monoclonal antibody B72.3 seemed to indicate protein, in addition to carbohydrate, as forming part of the conformationally dependent TAG-72 determinant.

Equivalents

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

## CLAIMS

1. An isolated immunoreactive species of the antigen CA 125 having a molecular weight of about 200 kDa.

2. An isolated immunoreactive species of the antigen CA 125 having the following characteristics:
   a. a molecular weight of about 200kDa;
   b. a bouyant density of about 1.36 g/ml;
   c. a carbohydrate composition of about 24% (by mass);
   d. a carbohydrate composition of sialic acid, fucose, mannose galactose, N-acetyl-glucosamine, and N-acetyl galactosamine in a ration of about 3.6/0.4/3.0/6.6/5.8/2.2.
   e. reactivity with the antibody OC 125.

3. A method for isolating the antigen CA 125, comprising the steps of:
   a. obtaining the cell culture medium from a culture of cells which shed CA 125 into the medium;
   b. subjecting the medium to acid precipitation to provide an acid soluble and an acid insoluble fraction;
   c. recovering and neutralizing the acid soluble fraction;
   d. separating the CA 125 species in the acid soluble fraction from lower molecular



weight components of the fraction by molecular exclusion chromatography and recovering the CA 125 species;
   e. treating the recovered CA 125 species with a chaotropic agent to disrupt high molecular weight CA 125 species;
   f. separating the lower molecular weight CA 125 species by molecular exclusion chromatography in the presence of the chaotropic agent;
   g. recovering the eluted fraction containing CA 125 species;
   h. contacting the CA 125 species with an immunoadsorbent comprising an antibody which binds CA 125 coupled to a resin under condition which permits selective adsorbtion of CA 125 by the immunoadsorbent; and
   i. recovering the CA 125 from the immunoadsorbent.

4. A method of Claim 3, wherein the cells which shed CA 125 antigen are ovarian carcinoma cells.

5. A method of Claim 4, wherein the acid precipitation is performed with perchloric acid.

6. A method of Claim 5, wherein the molecular size exclusion chromatography of step d is performed on Sepharose 4B-CL resin.

7. A method of Claim 6, wherein the chaotropic agent is urea or guanidine-HCl.

8. A method of Claim 7, wherein the molecular size exclusion chromatography of step f is performed on Sepharose 6B resin.

9. A method of Claim 8, wherein the chaotropic agent is separated from the CA 125 species by dialysis after the molecular size exclusion chromatography.

10. A method of Claim 9, wherein the antibody which binds to CA 125 is the OC 125 antibody.

11. A method of isolating CA 125, comprising the steps of:

a. obtaining a cell-free supernatant from a culture of ovarian carcinoma cells;

b. acidifying the supernatant to precipitate protein;

c. separating the precipitated protein from the acid soluble fraction of the supernatant;

d. neutralizing the soluble fraction;

e. separating the high molecular weight ( 1000kD) CA 125 from the smaller molecular weight CA 125 species and from other components in the soluble fraction by molecular size exclusion chromatography;

f. treating the high molecular weight CA 125 species with urea to disrupt the high molecular weight species;



g. separating the CA 125 species by molecular exclusion chromatography on a resin which retains molecules in the 200kD range in the presence of urea; and

h. immunopurifying the CA 125 species.

12. A method of Claim 11, wherein the ovarian carcinoma cells are selected from the group consisting of OVCA 433, NIH: OVCAR-3, SK-OV-3, CAOV-3 and CAOV-4.

13. A method of Claim 11, wherein the cells are OVCA 433.

14. A method of Claim 11, wherein the supernatant is acidified with perchloric acid.

15. A method of Claim 11, wherein the molecular size exclusion chromatography of step e is performed on Sepharose 4B-CL resin.

16. A method of Claim 11, wherein the urea is about 6 molar.

17. A method of Claim 11, wherein the molecular size exclusion chromatography of step g is performed on Sepharose B resin.

18. A method of isolating CA 125 species of about 200kD molecular weight, comprising the steps of:

a. obtaining a cell free supernatant from a culture of ovarian carcinoma cells which shed CA 125 into the culture medium;

b. acidifying the supernatant with perchloric acid to precipitate protein;

c. removing precipitated protein and neutralizing the acid soluble fraction;

d. submitting the neutralized acid soluble fraction to molecular size exclusion chromatography on Sepharose CL-4B resin and recovering from the column the fraction (void volume) containing CA 125 activity;

e. treating the fraction containing CA 125 activity with urea at about 6M;

f. submitting the urea treated fraction to molecular size exclusion chromatography on Sepharose CL-6B in a buffer controlled Urea 6M and about 1% SDS and recovering the eluted fraction containing CA 125 activity;

g. removing the urea from the recovered fraction;

h. applying the fraction to an immunoaffinity column comprising OC 125 antibody coupled to protein A Sepharose via dimethyl-pimelimidate

i. eluting the CA 125 from the immunoaffinity column with diethylamine.



19. An immunoadsorbent for specific adsorbtion of antibody reactive with CA 125 comprising an isolated immunoreactive species of CA 125 antigen.

20. An immunogen composition for immunizing an animal against CA 125 antigen comprising isolated CA 125 antigen in a physiologically acceptable vehicle.

21. An immunogen composition of Claim 20, wherein the isolated CA 125 antigen is an immunoreactive species of CA 125 having a molecular weight of about 200 kDa.

22. The use of immunogen of Claim 20 for production of antibody against CA 125.

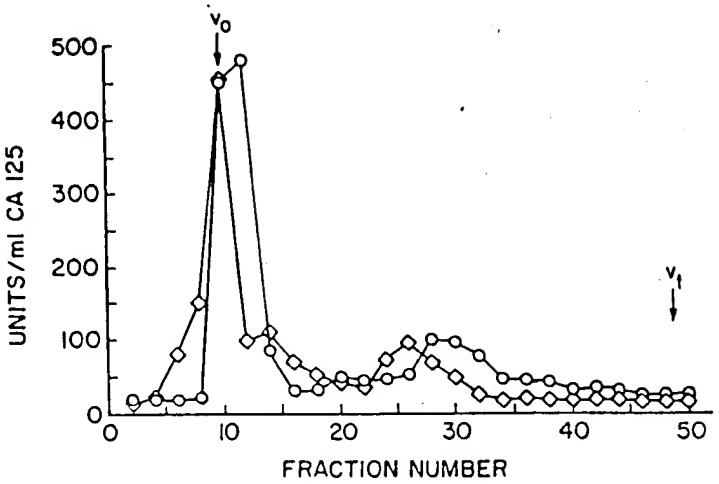



Fig. 1

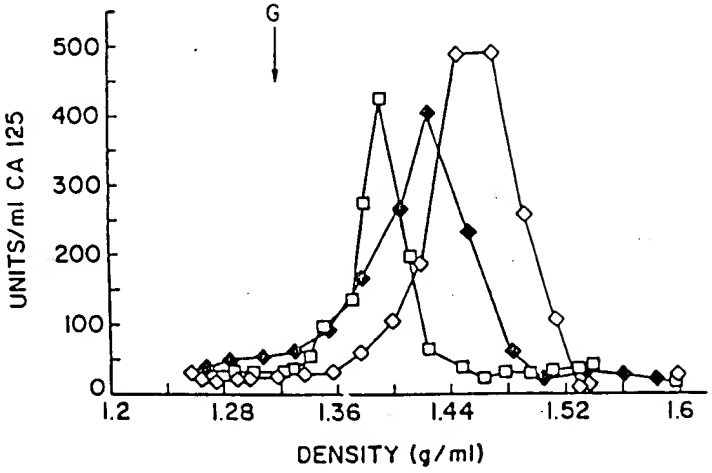



Fig. 2





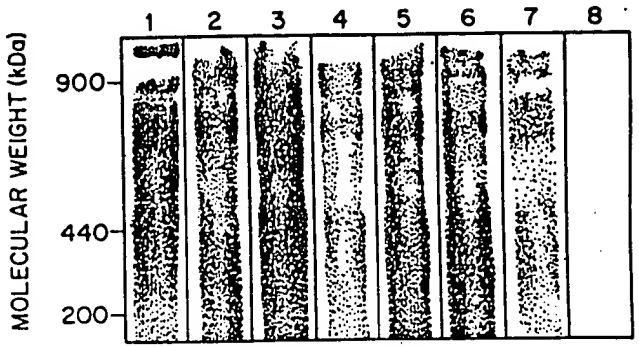



Fig. 3

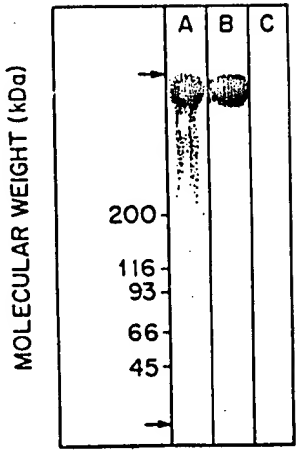
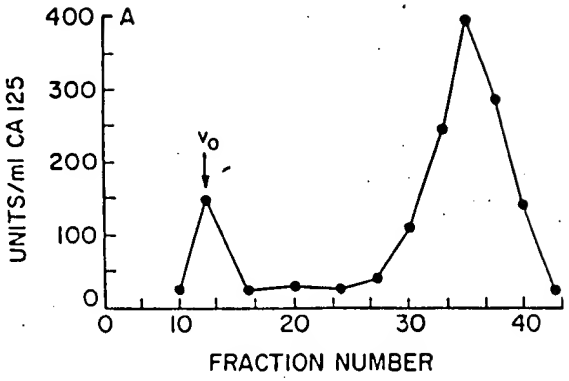



Fig. 4


A
400
300
200
100
0
$V_O$
UNITS/ml CA 125
0
10
20
30
40
FRACTION NUMBER

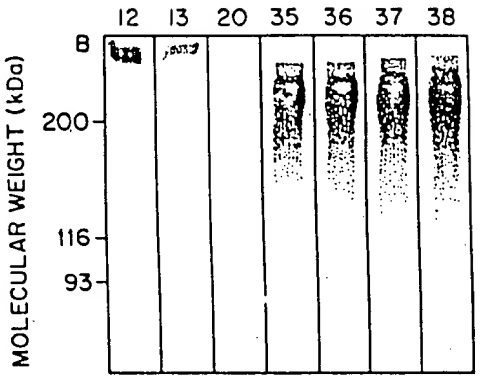

12
13
20
35
36
37
38
B
200
116
93
MOLECULAR WEIGHT (kDa)

Fig. 5





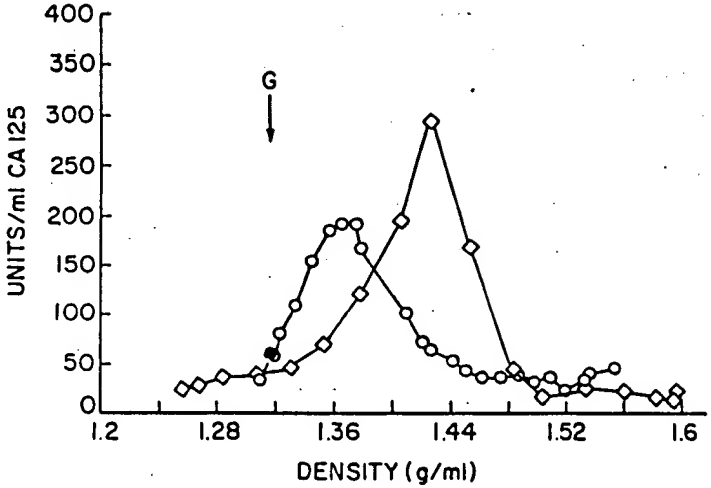

400
350
300
250
200
150
100
50
0
G
UNITS/ml CA 125
1.2
1.28
1.36
1.44
1.52
1.6
DENSITY (g/ml)

Fig. 6

# INTERNATIONAL SEARCH REPORT

International Application No PCT/US 87/03052

## I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [5]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC[4]: C 12 P 21/00; C 07 K 15/06; A 61 K 39/00

## II. FIELDS SEARCHED

Minimum Documentation Searched [7]

| Classification System | Classification Symbols |
|---|---|
| IPC[4] | A 61 K; C 12 P |

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

## III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

| Category [10] | Citation of Document, [11] with indication, where appropriate, of the relevant passages [12] | Relevant to Claim No. [13] |
|---|---|---|
| Y,P | Chemical Abstracts, volume 107, no. 19, 9 November 1987, (Columbus, Ohio, US), J.A Holt et al.: "Serum CA 125 and survival of mice inoculated with ovarian carcinoma and treated with antiestrogen, estrogen, or progestin", see page 81, abstract 169012u, & Gynecol. Oncol. 1987, 27(3), 282-93 -- | 1 |
| Y,P | Chemical Abstracts, volume 108, no. 3, 18 January 1988, (Columbus, Ohio, US), F. Leoni et al.: "Immunochemical characterization and radioimmunometric detection of molecules shed by human ovarian cancer", see abstract 20206y, & Int. J. Cancer 1987, 40(5), 592-7 -- | 1 |
| A | FR, A, 2317940 (MICROLOG LTD) 11 February 1977 -- | |
| A | US, A, 4584278 (SUZANNE KNAUF) 22 April 1986 | |

---

[10] Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

## IV. CERTIFICATION

| Date of the Actual Completion of the International Search | Date of Mailing of this International Search Report |
|---|---|
| 8th March 1988 | 18 APR 1988 |

International Searching Authority: EUROPEAN PATENT OFFICE

Signature of Authorized Officer: P.C.G. VAN DER PUTTEN

Form PCT/ISA/210 (second sheet) (January 1985)

# ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. US 8703052 SA 19823

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 08/04/88
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
|---|---|---|---|
| FR-A- 2317940 | 11-02-77 | DE-A- 2630380 | 03-02-77 |
| | | GB-A- 1554446 | 24-10-79 |
| | | JP-A- 53003509 | 13-01-78 |
| US-A- 4584278 | 22-04-86 | US-A- 4713351 | 15-12-87 |

For more details about this annex : see Official Journal of the European Patent Office, No. 12/82

This Annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 03/04/84

The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

| Patent document cited in search report | Publication date | Patent family member(s) | | Publication date |
|---|---|---|---|---|
| EP-A- 0062892 | 20/10/82 | JP-A- | 57179750 | 05/11/82 |
| | | AU-A- | 8256182 | 21/10/82 |
| WO-A- 8201773 | 27/05/82 | WO-A- | 8102899 | 15/10/81 |
| | | EP-A- | 0050129 | 28/04/82 |
| | | GB-A- | 2083836 | 31/03/82 |
| | | EP-A- | 0064063 | 10/11/82 |
| | | AU-A- | 7035781 | 26/10/81 |
| US-A- 4361647 | 30/11/82 | None | | |
| EP-A- 0042755 | 30/12/81 | WO-A- | 8200058 | 07/01/82 |
| | | GB-A- | 2107053 | 20/04/83 |
| | | EP-A- | 0044219 | 20/01/82 |
| | | WO-A- | 8200364 | 04/02/82 |
| EP-A- 0048357 | 31/03/82 | JP-A- | 57079455 | 18/05/82 |
| | | SE-A- | 8006424 | 13/03/82 |
| US-A- 4016043 | 05/04/77 | None | | |

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

| Category * | Citation of Document, [16] with indication, where appropriate, of the relevant passages [17] | Relevant to Claim No [18] |
|---|---|---|
| Y | EP, A, 0042755 (UNILEVER PLC) 30 December 1981<br>see examples; claims 1,2,3,8,9 | 1-9,12-20 |
| Y | EP, A, 0048357 (LA JOLLA CANCER RESEARCH FOUNDATION) 31 March 1982<br>see the entire document | 1-14 |
| Y | Gastroenterology, vol. 79, no. 5, point 2, published in 1980; J.R. Wands et al. "Immunodiagnosis of hepatitis B by high affinity monoclonal anti-HB antibodies", see page 1063 | 1-14 |
| A | US, A, 4016043 (A.H.W.M. SCHUURS et al.) 5 April 1977<br>see the entire document | 1-20 |

# INTERNATIONAL SEARCH REPORT

International Application No PCT/US 83/01147

## I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all) [3]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC[3]: G 01 N 33/54

## II. FIELDS SEARCHED

Minimum Documentation Searched [4]

| Classification System | Classification Symbols |
|---|---|
| IPC[3] | G 01 N |

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [5]

## III. DOCUMENTS CONSIDERED TO BE RELEVANT [14]

| Category * | Citation of Document, [16] with indication, where appropriate, of the relevant passages [17] | Relevant to Claim No. [18] |
|---|---|---|
| X | EP, A, 0062892 (AMERICAN HOECHST CORP.) 20 October 1982 see the entire document | 1-9,11-20 |
| X | WO, A, 82/01773 (CELLTECH LIMKED) 27 May 1982 see the entire document | 1-3,5-7,9-13 15,17,19 |
| X | Clinical Chemistry, vol. 29, no. 1, published in January 1983 (Washington, US) G. De Groote et al. "Use of monoclonal antibodies to detect human placental alkaline phosphatase", see pages 115-119 | 1-3,5-7,9, 10,12,13 |
| X | US, A, 4361647 (J. REMINGTON et al.) 30 November 1982 see columns 2-4; example 2; claims 1,3,5 & 6 | 1-10,12-14 |
| | | ./. |

* Special categories of cited documents: [15]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

## IV. CERTIFICATION

| Date of the Actual Completion of the International Search [2] | Date of Mailing of this International Search Report [2] |
|---|---|
| 20th March 1984 | 19 AVR. 1984 |
| **International Searching Authority [1]** | **Signature of Authorized Officer [20]** |
| EUROPEAN PATENT OFFICE | G.L.M. Kruydenberg |

042498

10

# CA 125 ANTIGEN IN HUMAN AMNIOTIC FLUID AND FETAL MEMBRANES

TIMOTHY J. O'BRIEN, Ph.D.,
JAMES W. HARDIN, Ph.D.,
GARY A. BANNON, Ph.D.,
JAMES S. NORRIS, Ph.D.,
and
J. GERALD QUIRK, JR., M.D., Ph.D.,
Little Rock, Arkansas

From the Departments of Obstetrics and Gynecology, Biochemistry, and Medicine, University of Arkansas Medical School.

Reprinted from
AMERICAN JOURNAL OF OBSTETRICS AND GYNECOLOGY,
St. Louis

Vol. 155, No. 1, pp. 50-55, July, 1986

# CA 125 antigen in human amniotic fluid and fetal membranes

**Timothy J. O'Brien, Ph.D., James W. Hardin, Ph.D., Gary A. Bannon, Ph.D., James S. Norris, Ph.D., and J. Gerald Quirk, Jr., M.D., Ph.D.**

*Little Rock, Arkansas*

The cancer antigen CA 125 is manifest by serous cystadenocarcinoma of the ovary and to a lesser extent by other gynecologic and nongynecologic tumors. Its presence was screened for in normal human fetal tissues and fluids. Appreciable quantities of CA 125 were discovered in amniotic fluid by both a dot blot assay and the commercially available immunoradiometric assay kit. The most likely source of this antigen was found not to be the developing fetus, since antigen was absent from cord blood and fetal urine, but rather the chorionic membrane, which contained significant quantities of the antigen. CA 125 was found in extracts of maternal decidua, but none was found in extracts of placenta or amnion. The CA 125 antigen was determined by gel filtration experiments to be in excess of 700,000 daltons and probably in the range of 2 to 3 $\times$ $10^6$ daltons. Size heterogeneity based on gel filtration and anion heterogeneity based on anion exchange chromatography have both been demonstrated for the CA 125 molecule. The amniotic fluid antigen is composed of two subunits of approximately 240,000 and 180,000 daltons as detected by iodine 125-labeled OC 125 monoclonal antibody. The antigen may contain additional subunits not detected by the monoclonal antibody. Size and change heterogeneity as well as the poor definition of the subunit bands on polyacrylamide gels also suggest this molecule contains an appreciable carbohydrate component. (Am J Obstet Gynecol 1986;155:50-5.)

**Key words:** CA 125, human, amniotic fluid, amnion, chorion, decidua

The cancer antigen CA 125 has been established as a tumor marker for serous cystadenocarcinomas of the ovary.[1-4] The antigen was discovered by use of a monoclonal antibody screen of hybridomas after stimulation of mice with cells derived from an ovarian cancer cell line, OVCA 433.[5] The monoclonal antibody found to be most useful was named OC 125. The antigen specified by OC 125 is a glycoprotein of high molecular weight (over 200,000) that has been partially purified from tissue culture media.[6] CA 125 antigen has been shown to be present in the sera of 85% to 90% of patients with ovarian carcinoma, 58% of patients with pancreatic carcinoma, 32% of patients with carcinoma of the lung, 22% of patients with carcinoma of the colon, and 30% of patients with miscellaneous gastrointestinal tract tumors.[1]

Although rather extensive data have been collected

*From the Departments of Obstetrics and Gynecology, Biochemistry, and Medicine, University of Arkansas Medical School.*

*Received for publication September 24, 1985; revised January 31, 1986; accepted February 12, 1986.*

*Reprint requests: Timothy J. O'Brien, Ph.D., Department of Obstetrics and Gynecology, University of Arkansas Medical School, 4301 W. Markham, Little Rock, AR 72205.*

that quantify CA 125 in sera from patients with malignant disease and from normal patients, little is known about the nature of the CA 125 antigen. Present data indicate the antigen is present on the cell surface of many cell lines of ovarian serous cystadenocarcinoma origin.[6] It likely is sloughed or secreted from the surface of malignant ovarian cells, thus accounting for its presence in sera of patients with serous ovarian carcinoma. It generally is not detected in tissues of normal adults. However, when a biotin-avidin immunoperoxidase staining technique was used, trace amounts of CA 125 were identified in the fallopian tube, endometrium, and endocervix. As a result, it has been suggested that CA 125 is an antigenic determinant associated with müllerian differentiation. In addition, the antigen was identified by similar assay in fetal and adult serosal surface epithelium incluing pleura, peritoneum, pericardium, umbilical epithelium, and amnion.[7] Recently Niloff et al.[8] have described the presence of CA 125 in amniotic fluid.

The present investigation was undertaken to identify a readily available source of CA 125 so that its biochemical characteristics could be better described and factors controlling the expression of this antigen might be elucidated. In this report we characterize normal fetal tissue and fluids as a source of CA 125 in large quantity and describe its expression and distribution during normal development.

## Material and methods

**Tissues and specimens.** Amniotic fluid obtained for amniocentesis was centrifuged; the fluid supernatant was collected and stored at −20° C until assayed. Cord blood, gastric aspirates, and fetal urine were obtained after normal delivery. Bloods were stored at −20° C as serum after clot removal. Fluids were stored directly at −20° C. Membranes, placenta, and decidual tissues were all obtained at the time of delivery, placed in ice-cold phosphate-buffered saline solution, and transported immediately to the laboratory. Blood clots were removed from tissues with several washes of cold phosphate-buffered saline solution. Amnion was dissected from chorion as a discrete sheet; the plane of cleavage was found routinely to be between the spongy layer of amnion and the cellular layer of chorion. These tissues were stored separately at −70° C.

**CA 125 assay.** All assays were carried out with use of a CA 125 assay kit purchased from Centocor Inc., Malvern, Pennsylvania, according to kit instructions. This immunoradiometric assay was first described by Klug et al.[9]

**Exclusion chromatography.** Initial preparation of amniotic fluid for chromatography was accomplished by filtering the fluid through a 1.25 μm filter followed by a fivefold concentration of the filtrate. Initially 10 ml of concentrate were applied to a 2 × 100 cm column containing HW 55 exclusion gel (fractogel HW 55 F) equilibrated with 20 mmol/L of bis-Tris-propane, pH 7.2, containing 0.15 mol/L of sodium chloride. The column was run at 0.5 ml/min and 5 ml fractions were collected. In subsequent experiments, 20 ml of sample was applied to a similarly prepared 5 × 100 cm column containing HW 65 exclusion gel (fractogel HW 65 F) and 9 ml fractions were collected.

**Fast protein liquid chromatography.** Fractionation of amniotic fluid was accomplished by applying a 500 μl sample to a mono Q anion exchange column (Pharmacia) and eluting the sample with a linear gradient of 0.015 mol/L of sodium chloride to 1.5 mol/L of sodium chloride containing 20 mmol/L of bis-Tris-propane, pH 7.2. The gradient control and the flow rate (1 ml/min) were effected by an LCC automated chromatography control unit (Pharmacia); 1 ml fractions were collected.

**Tissue extraction.** Tissues stored at −70° C were minced, after thawing, in 4 ml of phosphate-buffered saline solution per gram of tissue. The minced tissue was then homogenized with use of 5 by 5 second bursts on a Tekmar Tissuemiser. The homogenate was centrifuged at 10,000 × g for 20 minutes at 4° C and the supernatant assayed for CA 125.

**Dot blots analysis.** Dot blotting was carried out by transferring 150 μl aliquots of fractions collected from anion exchange chromatography to nitrocellulose filters under low vacuum in a micro sample filtration manifold (Schleicher & Schuell Inc.). Nitrocellulose filters were then washed with 10 ml of blotto (5% weight per volume of milk powder in phosphate-buffered saline solution) by shaking in a sealed plastic bag for 30 minutes. Washed filters were then exposed to $^{125}$I-labeled OC 125 (the monoclonal antibody for CA 125 labeled with radioactive $^{125}$I) at a concentration of $10^6$ cpm per 10 ml overnight. Nonspecific binding was reduced by washing the filters three times in 10 ml aliquots of blotto. Filters were exposed to x-ray film at −70° C for 2 days and developed in an automatic x-ray developer unit.

**Sodium dodecyl sulfate polyacrylamide gels.** Gradient polyacrylamide gels (9% to 15%) were run according to Lammli.[10] Protein preparations were first reduced and dispersed by incubation in sample buffer (60 mmol/L of tris, pH 6.8, 0.05% mercaptoethanol, 3.0% sodium dodecyl sulfate) at 100° C for 2 minutes; 25 μl aliquots were then electrophoresed for 4 hours at 30 m amp. After electrophoresis the polyacrylamide gel was equilibrated with Tris glycine transfer buffer (25 mmol/L of Tris, Ph 8.3, 192 mmol/L of glycine) and transferred to nitrocellulose filters by means of a transblot cell (Biorad Inc) in the presence of 20% methanol at 30 Volts overnight. Filters were then exposed to $^{125}$I antibody OC 125 in blotto as described for dot blots.

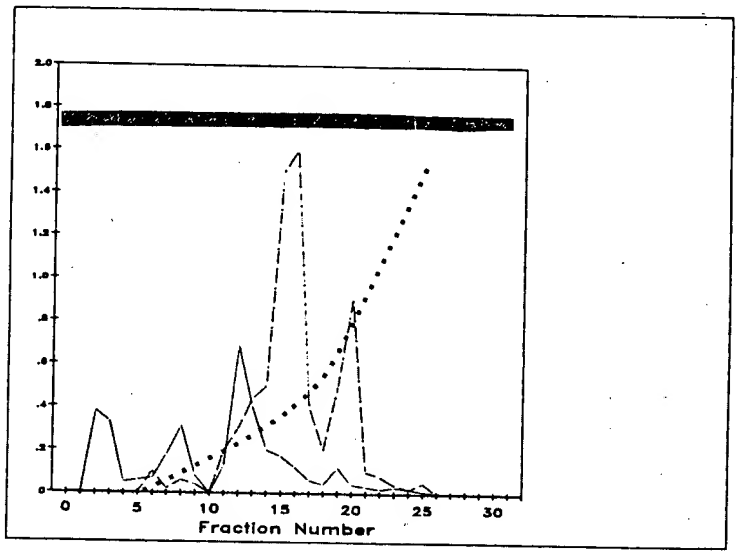



**Fig. 1.** Fast protein liquid *(FPLC)* anion exchange chromatography of amniotic fluid. Five hundred microliters of amniotic fluid was filtered through a 1.25 μm millipore filter before chromatography at Ph 7.2 on a mono Q anion exchanged column (Mono Q, FPLC, Pharmacia Inc.) with use of a sodium chloride gradient (■■) from 0.015 to 1.5 mol/L. One milliliter fractions were collected and assayed for CA 125 (-●-, cpm $\times 10^{-3}$) using a monoclonal immunoradiometric assay produced by Centocor Inc., Pennsylvania, or 150 μl was dot blotted to nitrocellulose filters, incubated with $^{125}$I-labeled OC 125 antibody, and exposed to x-ray film. ——, Optical density.

**Table I.** CA 125 in amniotic fluid

| *Specimen No.* | *Gestational age (wk)* | *CA 125 (U/ml)* |
|---|---|---|
| 1 | 13 | 7,375 |
| 2 | 15 | 7,450 |
| 3 | 15 | 9,875 |
| 4 | 15.5 | 4,750 |
| 5 | 16 | 4,500 |
| 6 | 16 | 7,450 |
| 7 | 16.5 | 13,000 |
| 8 | 16.5 | 7,200 |
| 9 | 17 | 3,250 |
| 10 | 17 | 5,250 |
| 11 | 18 | 7,750 |
| 12 | 18 | 4,450 |
| 13 | 19 | 9,500 |
| 14 | 20 | 6,050 |
| 15 | 26 | 5,750 |

## Results

Human amniotic fluid obtained throughout pregnancy has proved to be a rich source of CA 125 antigen. When amniotic fluid was filtered and then chromatographed on an anion exchange column with use of a sodium chloride gradient (0.015 to 1.5 mol/L), both the immunoradiometric assay system and a Western dot blot assay identified the presence of CA 125 in the same fractions (Fig. 1). Levels of CA 125 of 800 to 1000 U/ml have been observed by Kabawat et al.[4] in sera from patients with extensive tumor burden. Amniotic fluid from normal gestations has now been shown to have even higher CA 125 concentration (Table I).

**Table II.** Amniotic fluid CA 125 in twin pregnancies

| *Twin pregnancy No.* | *Twin infant* | *Gestational age (wk)* | *CA 125 U/ml* |
|---|---|---|---|
| 1 | A | 17 | 5,875 |
| | B | | 6,870 |
| 2 | A | 26 | 10,000 |
| | B | | 11,000 |
| 3 | A | 35 | 7,250 |
| | B | | 7,000 |
| 4 | A | Term | 2,000 |
| | B | | 1,900 |

Although a large number of amniotic fluid samples have not been assayed thus far, we have found no striking relationship between CA 125 concentrations and length of gestation. A wide range in CA 125 concentrations seems to be typical. Such also seems to be the case in twin gestations. In four pairs of amniotic fluid specimens from twin pregnancies, no differences in CA 125 concentrations were found between twins A and B (Table II) even though a wide range of concentration of CA 125 was observed among individual twin pregnancies.

We next sought to identify the source and distribution of CA 125 in human pregnancy. Serum obtained from normal pregnant women at various gestational ages (range, 10 to 40 weeks) had an average concentration of CA 125 of only 30 U/ml. Because significant

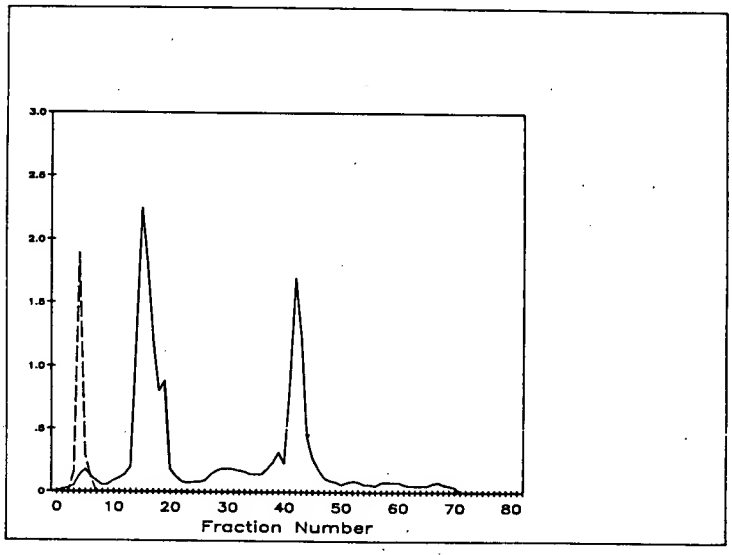



**Fig. 2.** Fractogel HW 55 F (E. M. Reagents) gel filtration chromatography of amniotic fluid. Fifty milliliters of amniotic fluid were concentrated fivefold in dialysis tubing with use of Aquaside II (Calbiochem). A fluid volume of 10 ml was layered over a 2 × 100 cm column of HW 55 F (fractionation range 10,000 to 700,000 daltons) and chromatographed at a flow rate of 0.5 ml/min. Five milliliter fractions were collected and assayed for CA 125 activity (- - - -, cpm × $10^{-4}$). ——, Optical density.

**Table III.** CA 125 in maternal and fetal body fluids

| *Fluid* | *No. of samples* | *CA 125 U/ml* |
|---|---|---|
| Cord serum | 6 | 17 |
| Fetal urine | 4 | 25 |
| Gastric aspirate | 6 | 4510 |
| Maternal serum | 9 | 28 |

**Table IV.** CA 125 in trophoblast and maternal tissues

| | *CA 125 (U/gm of tissue)* |
|---|---|
| Placental amnion | 255 |
| Extra placental amnion | 330 |
| Placental chorion | 41,749 |
| Extra placental chorion | 44,930 |
| Placenta | 248 |
| Decidua | 29,411 |

quantities of CA 125 were present in amniotic fluid at term, we measured concentrations of the antigen in cord blood in first-voided neonatal urine specimens, and in gastric aspirates of newborn infants in a preliminary attempt to identify the source of this CA 125. Neither cord blood nor first neonatal urine had significant concentrations of CA 125 (Table III). In contrast, gastric aspirates contained significant concentrations of CA 125, but these levels did not differ from levels found in amniotic fluid and therefore were probably derived from amniotic fluid.

To evaluate further the source of CA 125 in amniotic fluid, CA 125 was assayed in tissue extracts of fetal membranes, placenta, and maternal decidua vera from women undergoing elective cesarean delivery. The amniotic membranes were totally separated from the chorion and divided into two categories (1) membrane overlying the placenta (placental amnion and placental chorion) and (2) membrane surrounding the remainder of the gestational sac (extraplacental amnion and extraplacental chorion). Compared to amnion and placenta, there were extraordinary quantities of CA 125 in the 10,000 × g supernatant of extracted chorionic membrane. No "geographic" differences in the distribution of CA 125 within chorion were observed; that is to say, extracts of chorion overlying the gestational sac and extracts of chorion overlying placenta had similar concentrations of antigen in their 10,000 × g fractions (Table IV). Significant quantities of CA 125 were recovered for the 10,000 × g supernatant fraction of decidua. It is yet to be determined whether this represents a synthetic source of CA 125 or whether this represents contamination from the overlying chorion.

CA 125 eluted in the void volume of a gel matrix designed to fractionate molecules up to 700,000 daltons, indicating that CA 125 is totally excluded from the matrix of that column (Fig. 2). Further chromatography of antigenic activity over a second exclusion matrix that is designed to separate molecules in the range of 5 million to 50,000 daltons indicated that this

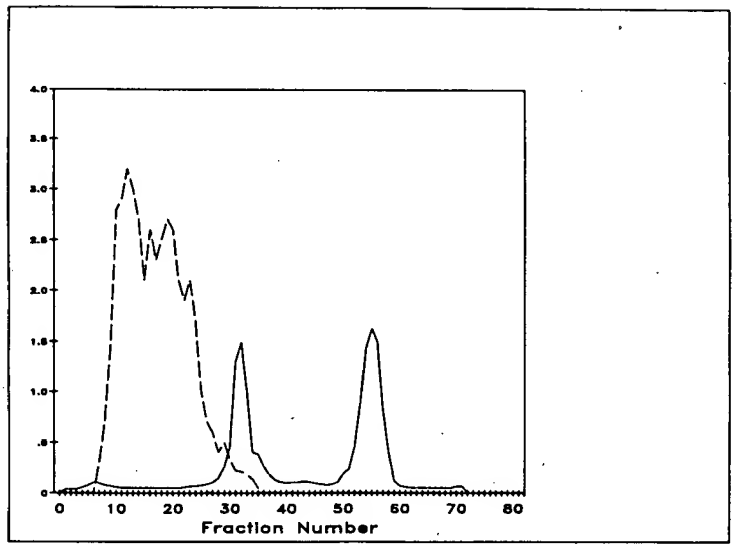



**Fig. 3.** Fractogel HW 65 F (E. M. Reagents) gel filtration chromatography of amniotic fluid. Amniotic fluid was prepared as described in Fig. 2. Twenty milliliters of 5× concentrated amniotic fluid were applied to a 5 × 100 cm column of HW 65 F (fractionation range 50,000 to 5,000,000 daltons). Nine milliliter fractions were collected and assayed for CA 125 activity (- - - -, cpm × $10^{-3}$). ——, Optical density.

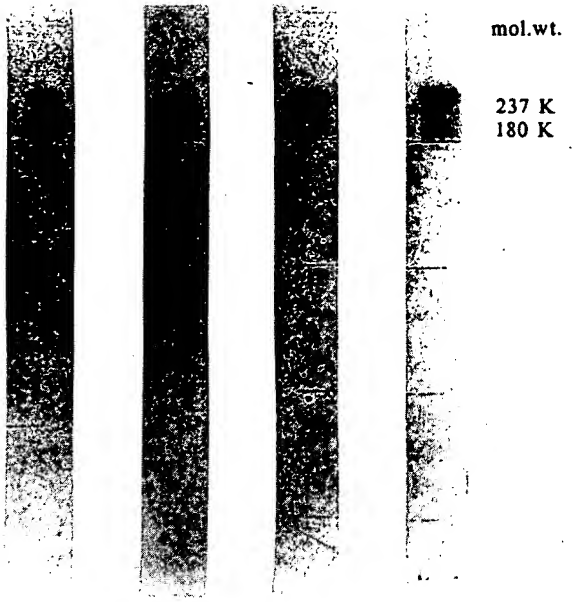



**Fig. 4.** Sodium dodecyl sulfate polyacrylamide gel electrophoresis of amniotic fluid CA 125. Twenty-five microliter aliquots of amniotic fluid were electrophoresed over a 9% to 15% polyacrylamide gradient gel after dispersion in sodium dodecyl sulfate and mercaptoethanol. After electrophoresis, proteins were transblotted electrophoretically to a nitrocellulose filter, and the filter was probed with $^{125}$I-labeled OC 125 antibody. Bound radioactivity was determined by exposing the filters to x-ray film. Molecular weights were determined by cochromatography of molecular weight standards after removal of the gel lanes containing the standards, followed by Coomassie blue staining.

antigen coeluted in the range of blue dextran and showed considerable size heterogeneity (Fig. 3). Sodium dodecyl sulfate–polyacrylamide gel electrophoresis analysis of CA 125 followed by transblotting to nitrocellulose and probing with $^{125}$I-OC 125 antibody indicated two major bands of antigenic binding at approximately 186,000 and 237,000 daltons (Fig. 4). It was further noted that on several occasions these bands were more diffuse or less discrete.

## Comment

A significant concentration of the CA 125 antigen has been found in amniotic fluid, which is equivalent to the highest concentrations found in the sera of patients with a large tumor burden. The most likely source of this antigen is suspected to be the chorion, since very little CA 125 was detected in fetal serum or urine. Significant concentrations of antigen were discovered in decidual tissues, but little or no antigen was discovered in amnion or placental extracts. Levels of antigen were highest in extracts of chorion and as such the possibility of decidual contamination with CA 125 from chorion is possible. The nature of the CA 125 molecule is unusual in several ways. As indicated in Fig. 2, CA 125 is an extraordinarily big molecule, at least as indicated by molecular sieving chromatography in which profiles suggest molecular size as high as 2 to 3 × $10^6$ daltons with notable size heterogeneity (Fig. 3). Both the size and the molecular heterogeneity point to a glycoprotein structure with major polysaccharide content. Subunit

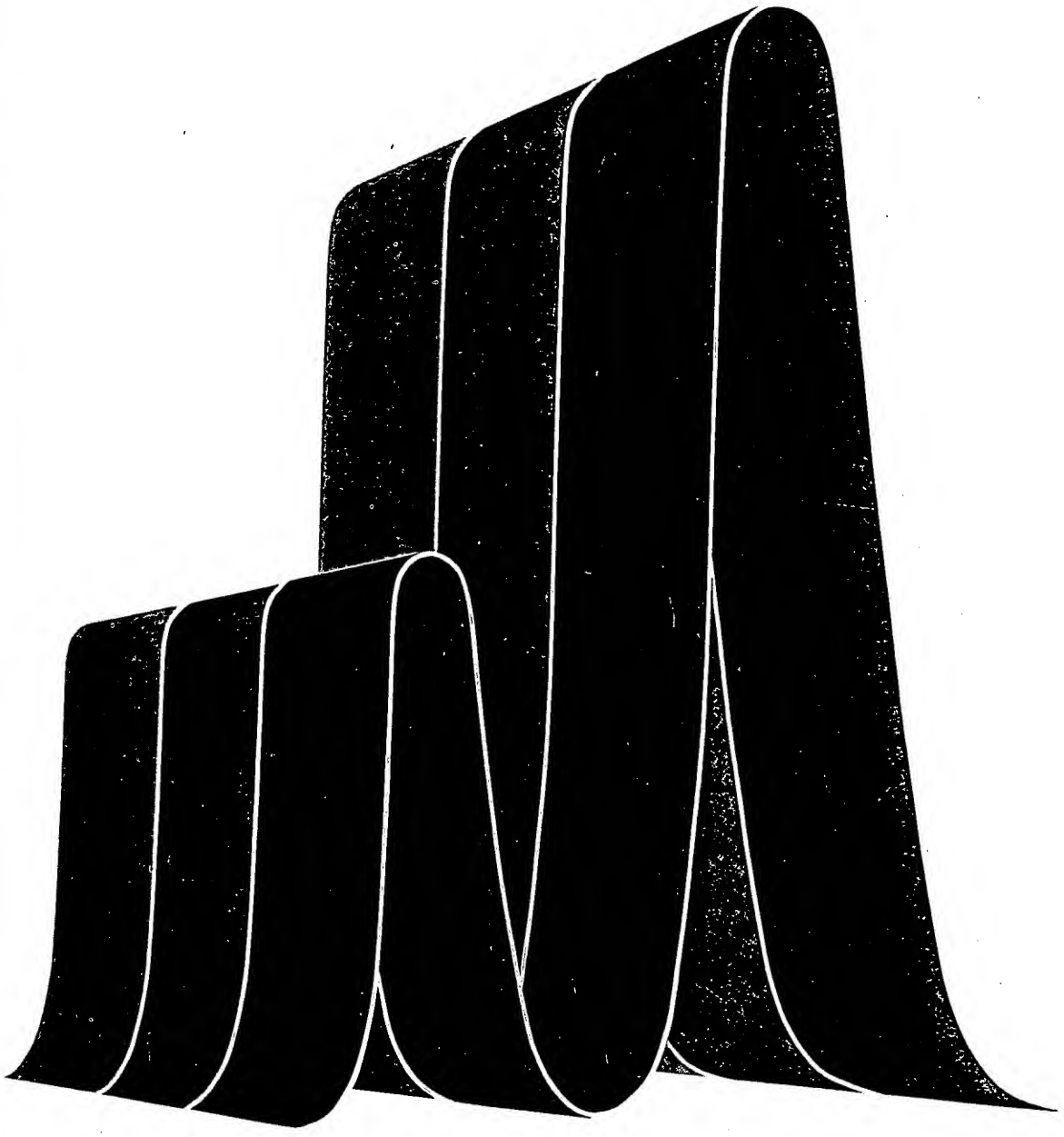
analysis of CA 125 on sodium dodecyl sulfate reducing gels indicated the presence of two subunits (237,000 and 186,000 daltons). Because both subunits are detected by a monoclonal antibody (OC 125) these data indicate the presence of shared antigenetic determinants but do not preclude the presence of other subunits not recognized by the monoclonal antibody. It is also possible that the lower molecular weight subunit (186,000 daltons) is a breakdown product of the 237,000 molecular weight species and therefore contains the same antigenic determinant. The diffuse pattern of the bands detected by the monoclonal antibody is typical for glycopeptides and would further implicate a carbohydrate nature to the CA 125 molecule. The detection and partial characterization of the CA 125 molecule synthesized during normal fetal development by trophoblast-derived chorionic cells provides a foundation for an evaluation of the expression of this gene in normal and transformed tissues. The detailed structure, function, and expression of the CA 125 molecule in normal gestation is presently under investigation.

## REFERENCES

1. Bast RC, Klug TL, St John E, et al. A radioimmunoassay using a monoclonal antibody to monitor the course of epithelial ovarian cancer. N Engl J Med 1983;309:883-7.
2. Bast RC, Klug TL, Schaetzl EM, et al. Monitoring human ovarian carcinoma with a combination of CA 125, CA 19-9, and carcinoembryonic antigen. Am J Obstet Gynecol 1984;149:553-9.
3. Niloff JM, Bast RC, Schaetzl EM, Knapp RC. Predictive value of CA 125 antigen levels in second-look procedures for ovarian cancer. Am J Obstet Gynecol 1985;151: 981-6.
4. Kabawat SE, Bast RC, Welch WR, Knapp RC, Colvin RB. Immunopathologic characterization of a monoclonal antibody that recognizes common surface antigens of human ovarian tumors of serous, endometrioid, and clear cell types. Am J Clin Pathol 1983;79:98-104.
5. Bast RC, Feeney M, Lazarus H, Nadler LM, Colvin RB, Knapp RC. Reactivity of a monoclonal antibody with human ovarian carcinoma. J Clin Invest 1981;68:1331-7.
6. Masuho Y, Zalutsky M, Knapp RC, Bast RC. Interaction of monocolonal antibodies with cell surface antigens of human ovarian carcinomas. Cancer Res 1984;44:2813-9.
7. Kabawat SE, Bast RC, Bhan AK, Welch WR, Knapp RC, Colvin RB. Tissue distribution of a coelomic-epithelium-related antigen recognized by the monoclonal antibody OC125. Int J Gynecol Pathol 1983;2:275-85.
8. Niloff JM, Knapp RC, Schaetzl EM, Reynolds C, Bast RC. CA125 antigen levels in obstetrics and gynecologic patients. Obstet Gynecol 1984;64:703-7.
9. Klug TL, Bast RC, Niloff JM, Knapp RC, Zurawski VR. Monoclonal antibody immunoradiometric assay for an antigenic determination (CA125) associated with human epithelial ovarian carcinomas. Cancer Res 1984;44:1048-53.
10. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970;227:680-5.

MLW 042498

# Gel filtration
## theory and practice



Pharmacia
Fine Chemicals

# Chromatographic properties

## Selectivity

Each Sephadex G-type has a different molecular weight range over which molecules can be fractionated. Molecules with molecular weight above the upper limit of this range, the exclusion limit, are totally excluded from the gel and elute at the void volume. Molecules smaller than the lower limit are usually eluted at an elution volume approximately equal to the bed volume.

Table 1 gives the fractionation ranges for the different G-types. Figures 2 and 3 show the selectivity curves for globular proteins.

**Table 1.** Properties of Sephadex.

| Sephadex type and grade | | Dry bead diameter μm | Fractionation range (molecular weight) | | Bed volume ml/g dry Sephadex |
|---|---|---|---|---|---|
| | | | Peptides and globular proteins | Dextrans | |
| G-10 | | 40—120 | — 700 | — 700 | 2—3 |
| G-15 | | 40—120 | — 1 500 | — 1 500 | 2.5—3.5 |
| G-25 | Coarse | 100—300 | 1 000— 5 000 | 100— 5 000 | 4—6 |
| | Medium | 50—150 | | | |
| | Fine | 20— 80 | | | |
| | Superfine | 10— 40 | | | |
| G-50 | Coarse | 100—300 | 1 500— 30 000 | 500— 10 000 | 9—11 |
| | Medium | 50—150 | | | |
| | Fine | 20— 80 | | | |
| | Superfine | 10— 40 | | | |
| G-75 | | 40—120 | 3 000— 80 000 | 1 000— 50 000 | 12—15 |
| | Superfine | 10— 40 | 3 000— 70 000 | | |
| G-100 | | 40—120 | 4 000—150 000 | 1 000—100 000 | 15—20 |
| | Superfine | 10— 40 | 4 000—100 000 | | |
| G-150 | | 40—120 | 5 000—300 000 | 1 000—150 000 | 20—30 |
| | Superfine | 10— 40 | 5 000—150 000 | | 18—22 |
| G-200 | | 40—120 | 5 000—600 000 | 1 000—200 000 | 30—40 |
| | Superfine | 10— 40 | 5 000—250 000 | | 20—25 |

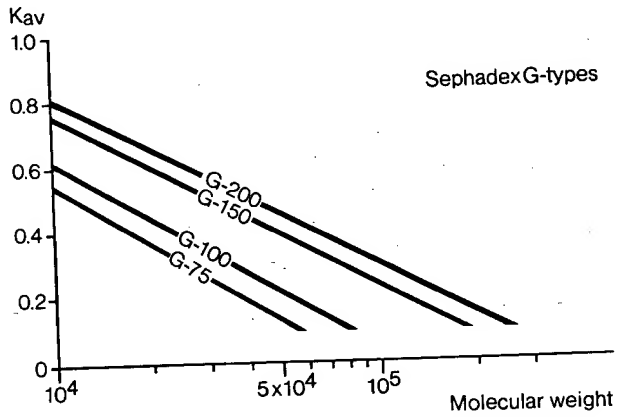



**Fig. 2.** Selectivity curves: Sephadex G-types (globular proteins).